The cover features a photograph of two butterflies with intricate orange, black, and white patterns on their wings. They are perched on a vertical spike of small, light purple flowers. The background is a soft, out-of-focus green. On the left side, there is a vertical bar with an orange top section and a teal bottom section.

Jan Christian Habel  
Thorsten Assmann  
*Editors*

# Relict Species

Phylogeography  
and Conservation Biology

 Springer

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Jan Christian Habel • Thorsten Assmann  
Editors

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 Fonds National de la  
Recherche Luxembourg

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ISBN 978-3-540-92159-2                      e-ISBN 978-3-540-92160-8

DOI 10.1007/978-3-540-92160-8

Springer Heidelberg Dordrecht London New York

Library of Congress Control Number: 2009932420

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# Foreword

Mankind has evolved both genetically and culturally to become a most successful and dominant species. But we are now so numerous and our technology is so powerful that we are having major effects on the planet, its environment, and the biosphere. For some years prophets have warned of the possible detrimental consequences of our activities, such as pollution, deforestation, and overfishing, and recently it has become clear that we are even changing the atmosphere (e.g. ozone, carbon dioxide). This is worrying since the planet's life systems are involved and dependent on its functioning. Current climate change – global warming – is one recognised consequence of this larger problem.

To face this major challenge, we will need the research and advice of many disciplines – Physics, Chemistry, Earth Sciences, Biology, and Sociology – and particularly the commitment of wise politicians such as US Senator Al Gore.

An important aspect of this global problem that has been researched for several decades is the loss of species and the impoverishment of our ecosystems, and hence their ability to sustain themselves, and more particularly us! Through evolutionary time new species have been generated and some have gone extinct. Such extinction and regeneration are moulded by changes in the earth's crust, atmosphere, and resultant climate. Some extinctions have been massive, particularly those associated with catastrophic meteoric impacts like the end of the Cretaceous Period 65Mya. Each time, new species and ecosystems have evolved subsequently over millions of years. The rate of species loss today due to Man's activities is great, and has been classed as the next major extinction.

One way to study this problem is to focus on Relict Species. As species become rare through natural processes, they are reduced to few populations in few locations and are considered as threatened or endangered. Numerous species are now placed in this dangerous situation because of Man's expansion. This leads to great public concern and efforts for their conservation. To better inform these efforts, it is most valuable to study the origins, history, and status of such Relict Species.

For this, one clearly needs research on the species habitat requirements and ecological limits, particularly with the growing concern for current climate change. There is pertinent research on the last 2My, the Quaternary Period, with its cycles of increasingly severe ice ages that caused great changes in species abundance and distribution as seen in the fossil record. Our understanding of the origins and

history of species have been greatly advanced by new DNA methods that allow the genetic relations and diversity across a species range to be revealed and analysed. This Phylogeographic approach is informing and sometimes challenging the views in Biogeography and Conservation, and permeating most areas of Evolutionary Biology.

A symposium on Relict Species seemed a good way forward. This would bring together the people studying the various relevant aspects, including topics in Ecology, Phylogeography, Climate Change, and Conservation. And so one was organised in Luxembourg in 2007. Europe was the theatre concerning many participants, where there is considerable interest in ice age refugia and postglacial colonisation to produce our present biota. Studies from Africa, North and South America, and Asia were also included, and the range of organisms covered fungi, plants, many insects, reptiles, and mammals. The relevance of phylogeography and climate change to the conservation of species was addressed in many contributions.

Museums were often considered as dusty shrines. But today the major ones are concerned in modern research in the evolution and management of biodiversity and in educating the next generation in such important matters. They also serve as rich archives and sources of material for such studies. The organisation of this symposium by the Luxembourg Museum is a signal example of such farsighted and crucial involvement.

April 2009

Godfrey Hewitt

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# Relict Species: From Past to Future

Jan C. Habel, Thorsten Assmann, Thomas Schmitt,  
and John C. Avise

## 1 What Are Relicts?

Dictionaries define a “relict” as something that has survived, usually as a trace, from the past. In biology, relicts are distinctive populations or species that typically are small in size or severely restricted in geographic range. Biologists distinguish between taxonomic and biogeographic relicts. Taxonomic relicts are a few or sole survivors of a once diverse taxonomic assemblage, whereas biogeographic relicts are descendants of once widespread taxa (or populations) that now have a narrow geographic distribution (Lomolino et al. 2006). Both categories sometimes coincide, as for example in the case of “living fossils” (such as ginko, lungfishes, crossopterygians, or marsupials) that closely resemble their ancient ancestors in overall phenotype (Futuyma 2005; Lomolino et al. 2006; Beierkuhnlein 2007). In the following, we focus on biogeographic relicts.

Climatic and other large-scale environmental changes can have fundamental impact on the distributional patterns of species and alter the composition of communities and ecosystems (e.g. Hewitt 1999; Schweiger et al. 2009). Species react by range shifts, or by local or regional extinctions when their *adaptive capacity* is exceeded (Agarwal 2001). The origins and distributions of modern-day relicts can often be related to environmental changes of the past. Glacial periods and warm interglacial periods of the Quaternary, in particular, often help us to understand how relict species and

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populations arise and sometimes have served as important sources for re-colonizing much larger areas (de Lattin 1967). For example, many thermophilic species survived the last ice age in relatively small refugia on Europe's Southern peninsulas. During interglacial periods, these species often colonized large areas. Thus, many temperate species that are widespread today existed as small relict populations just a few thousand years ago (cf. Schmitt 2007). Conversely, many cold-adapted species show evidence of formerly wide distributions, followed by severe range restrictions during postglacial warming. Prominent examples of the latter are the butterflies *Procllossiana eunomia* pictured on this book (front side) and *Lycaena helle* (small picture on the back side), which currently resides only in high-elevation mountain enclaves and at more Northern latitudes that have been shown to coincide with distinctive genetic clusters in this species (Habel et al. 2009). Although such species today (and during the other warm periods between glacial periods) are restricted to small areas, they may have the potential to recolonize larger areas if and when the climate cools again. In general, the assumption that relict species inevitably are on their way to extinction may be erroneous; at least some relict populations undoubtedly retain the potential to adapt to a broad spectrum of environmental conditions (Hampe and Petit 2005).

Range restrictions and expansions are also known to result from human-mediated landscape changes. For example, woodlands covered large areas of Central Europe prior to anthropogenic deforestation. Especially during the Middle Ages and early modern times, these extensive woodlands were degraded to a few small remnants. After the establishment of modern forestry and changes in land use, woodland coverage again increased enormously. The former woodland remnants preserved forest-inhabiting plants and animals, some of which still show relict-like distributions but others of which were able to re-colonize large areas (e.g. Assmann 1999; Drees et al. 2008). Comparable changes are known for numerous habitat types and regions. These alternating scenarios of range expansions and restrictions remind us that species with relict-like versus wide distributions are *not* necessarily contradictory indicators of past environmental changes.

## 2 Conservation of Relict Species and Populations

Why focus special conservation attention on relict as opposed to non-relict taxa? One explanation may reside in the connotation for relict that appears from the word "trace" in the dictionary definition. Typically, little remains of any relict, implying in the current biological context that extant populations of a relict species are few in number or small in size. Thus, populations of a relict taxon might occupy disjunct mountain peaks or perhaps a few isolated lakes, for example. Rarity and restricted distributions obviously place relict species in special demographic jeopardy of extinction, especially in the face of ongoing climatic changes and other ecological perturbations.

Another more subtle connotation in the dictionary definition is that relicts are unchanged or "left behind" relative to their non-relict counterparts. It seems doubtful,

however, that evolutionary genetic change has ceased or even slowed substantially in relict species. Indeed, if genetic drift is a potent evolutionary force (as expected for small populations), then the genetic differences of extant relicts from their ancestors or from their modern sister taxa might even be greater than the genetic differences of non-relict species from their respective ancestors or extant relatives. One of the most robust discoveries from the field of molecular evolution is that genetic change proceeds inexorably through time, in essentially all species, and sometimes at surprisingly steady mean rates. An interesting scientific question is whether relict species (as defined by small population size or restricted geographic distribution) tend to evolve at the genotypic or phenotypic levels at rates that differ consistently from those of their non-relict counterparts (García-Ramos and Kirkpatrick 1997).

Another set of scientifically interesting questions stems from the fact that various relict species and populations can have strikingly different ages, thus making them excellent objects for comparative evolutionary and ecological studies (Lesica and Allendorf 1995). In particular, phylogeographic surveys can give great insights into historical, ecological and evolutionary processes related to past shifts (restrictions and expansions) in the distributions of species (Avice 2000; Hewitt 2000, 2004; Schmitt 2007). Moreover, by helping to identify evolutionarily significant units (ESUs) and management units (MUs), phylogeographic and population genetic analyses can help to identify relict populations of special importance for conservation and management (cf. Moritz 1994; Avice and Hamrick 1996; Pérez-Tris et al. 2004; De Guia and Saitoh 2006). Finally, there is a huge scientific interest in stochastic processes as well as inbreeding in relict species that consist either of single populations or structured metapopulations (cf. Melbourne and Hastings 2008). These processes can reduce genetic variability (Petit et al. 2003; Chang et al. 2004) and diminish genetic fitness (cf. Berger 1990; Reed and Frankham 2003) within relict populations of both plants (Oostermeijer et al. 1996) and animals (Wynhoff et al. 1996; Madsen et al. 1999).

Many relict species are already endangered and referenced in *Red Lists*. Conservation must not ignore relicts, as they form an essential component of overall biodiversity. Relict populations and species are, almost by definition, “survivors”. Thus, it is both sad and ironic that conservation biologists must now be concerned about the survival into the future of relict species that, by definition, are proven survivors from the evolutionary past. Nonetheless, such conservation concern is merited, because human actions are precipitating global climatic and other environmental changes that may fall outside the adaptive scope of many relict (and other) living species.

### 3 The Scope of This Book

This book focuses on relict species and describes their history, current status, and future trends. It presents a compilation of case studies representing different methodological approaches and different temporal and spatial scales, all meant to

illustrate evolutionary processes and ecological traits of isolated remnant populations. The book is structured into five sections. Section 1 deals with the basics of climate change and the responses of species and ecosystems. Sections 2 and 3 deal with the effects of pre-glacial and glacial phenomena, and postglacial range expansions, on relict species. Section 4 includes conservation approaches to protect and preserve relict species. Finally, in Section 5, future trends of relict species and their projected distributional patterns are discussed. The book also includes four mini-reviews that highlight molecular techniques, the biogeography of Europe, cave species, and ecological niche modelling.

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# The Changing Climate: Past, Present, Future

Markus Quante

**Abstract** Over the 4.6 billion years of its existence, the Earth has seen a large variety of climate states. During the evolution of our planet, its climate was characterized by periods of enhanced climate variability or even swings and some more or less stable – almost quiet – periods. Natural climate variability was the rule rather than an exception and the evolution of life on Earth was closely linked to climate and its change.

For about 250 years, mankind has interfered stronger with the climate system via the release of radiative gases and particles in substantial amounts into the atmosphere. A global mean near surface temperature increase – global warming – can be deduced from instrumental observations, which started in about 1860. The pace and amount of this temperature increase is unprecedented at least the past 1600 years, as reconstructions from proxy data indicate. The observed warming can be attributed to a large extent to human activities as the most recent report of Intergovernmental Panel on Climate Change states. The atmospheric temperature increase is accompanied by an increase in sea surface temperature and a rise of the sea level. Evidence is building that human-induced climate change has also a direct influence on changes in precipitation and the hydrological cycle.

Climate projection driven by socio-economic scenarios indicate that the global temperature and sea level rise will continue throughout the twenty-first century and beyond, the amount of which is strongly dependent on the underlying emission assumptions.

There are a few climate elements that may be sensitive to sudden, abrupt changes, when a set of conditioning parameters is overstepped or certain thresholds are passed; a prominent example is a possible collapse of the thermohaline circulation in the North Atlantic. Here, further research is necessary to quantify thresholds, effects and time horizons.

Overall it can be said that a certain amount of future climate change is unavoidable regardless which route of emission reductions mankind will follow and that emissions from the twenty-first century will noticeably affect climate over the entire millennium.

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## 1 Introduction

About 4.6 billion years ago the solar system, including planet Earth, was formed. During the evolution of our planet its climate was characterized by periods of enhanced variability or even swings and some more or less stable – almost quiet phases. The history of the Earth is a story of the co-development of the oceans, the atmosphere, crustal rocks, climate and life in a cosmic perspective (Cloud 1978). From the viewpoint of us human beings, one of the most striking questions in this context is: what is needed and in what quality and quantity to enable planets to support life? Whether planets are suitable for life depends to a large extent on their volatile abundances, especially water, and, as a precondition for their climate which is strictly determined by the availability of energy, on the distance from their central star.

The Earth's climate has remained conducive to life for at least the past 3.5 billion years, despite a much lower solar luminosity at the beginning of this time span (about 30% less, Gough 1981), which is known as faint young sun (Kasting et al. 1988; Kasting and Catling 2003). The fact that the global temperature varied only between values which allowed for liquid water during the early phase (faint young sun paradox) is most probably due to elevated concentrations of the greenhouse gases carbon dioxide ( $\text{CO}_2$ ) and/or methane ( $\text{CH}_4$ ), which are involved in long-term feedback loops, which stabilize climate in such a way as to allow a water cycle to be maintained (Broecker 1985; Pavlov et al. 2000; Kasting 2005).

The main external and internal natural drivers for climate variability which had more or less an influence over the entire time span of the Earth's history are related to changes in the sun's output on different time scales (e.g. Haigh 2007), orbital changes with different periodicities (e.g. Berger 1988), plate tectonics (Raymo and Ruddiman 1992), and volcanism (Lamb 1970; Robock 2000). After a purely physicochemical determined phase at the very beginning of the evolution of life, the climate started to be strongly influenced by the biosphere. With the emergence of photosynthesis in the oceans and on land, the biosphere contributed essentially to the high oxygen concentration in the atmosphere and thereby gained an important influence on the chemistry of trace gases. The biosphere started to become involved in many of the cycles of matter, and with the water cycle and the carbon cycle established the strongest signature in the climate system. The development and expansion of vegetation on land substantially changed the albedo of the Earth's surface, thereby impacting on the energy budget of the planet.

The stability of climate over several recent millennia has allowed land to be cleared, developed and activated to allow for the production of food needed by a growing population. With the development of agriculture some 1,000 years ago and the related release of greenhouse gases, mainly  $\text{CO}_2$  and  $\text{CH}_4$ , and changes in land cover affecting the Earth's albedo and biogeophysical cycles, humans started to influence the global climate (Ruddiman 2003). With the advent of coal-burning factories and power plants in modern times, industrial societies began to release  $\text{CO}_2$  on a large scale into the atmosphere. The development of the mobile society with an ever increasing number of motorised vehicles (locomotives, ships and cars)

added to the emission portfolio. Crutzen and Stoermer (2000) called the industrial era with humans massively altering the greenhouse gas concentration in the atmosphere as the “Anthropocene”; the year 1800 has been assigned as the beginning of this epoch (see also Steffen et al. 2007). All relevant greenhouse gas concentrations have increased considerably over the last 200 years and the global mean temperature is rising and projected to rise throughout the next centuries (IPCC 2007a). Today, mankind finds itself in a position where urgent decisions need to be made and address the issues - in what form and amount emissions related to its activity have to be reduced - in order to *avoid* dangerous interferences with the climate system, as is postulated by the United Nations Framework Convention on Climate Change (UN 1992).

Stabilizing the carbon dioxide component of climate change is strongly related to changing *how* we produce our energy. Stabilization will require carbon emission-free primary energy sources in addition to a reduced end-use energy demand and improvements in energy efficiency. In a widely recognized paper, Pacala and Socolow (2004) build up a scenario with a target value of 500 ppm CO<sub>2</sub> over a 50-year-time scale, which relies entirely on current technologies. In contrast, Hoffert et al. (2002) conclude from their assessment that only an advanced technology path can lead to a stabilized climate and that intensive research and development on this sector is urgently needed. Besides mitigation, adaptation measures (IPCC 2007b) are also being discussed, given the prospect that a certain change of the global climate is almost unavoidable (IPCC 2007a). In recent years, an essentially different and older idea to address global warming has been reanimated, it concentrates on an active, deliberate *interference* with the climate system in order to reduce the risks associated with climate change. The proposed measures for an attempt to transform the Earth climate on planetary scale are generally summarised by the expression *geoengineering* (Keith 2000; Schneider 2001; Weart 2008). *Geoengineering* is currently being broadly but controversially discussed, since many consequences of the proposed concepts are highly uncertain, and might stay uncertain for a long time, and the ethical, political and legal aspects are only barely touched upon (Fleming 2007; Robock 2008; Schneider 2008).

Most probably, climate change *is* and *will be* affecting the physiology, phenology and the distribution of plant and animal species all over the world. Observational and modelling studies are supporting this statement, with details being still under discussion. Plants and animals are responding in an overall unsurprising way, as they did at the end of the last ice age. Mainly in an effort to keep the temperature zone they are adapted to (but other parameters might also be important), they are shifting their range towards the higher latitudes and, if possible, towards higher altitudes such as mountainous regions. Such shifts have been observed in a wide range of species. European butterflies can serve as a well-documented example, and Parmesan et al. (1999) found that more than 2/3 of the species they followed had shifted their range Northward during the twentieth century. In the Alps and the rocky mountains, trees and grasses have been migrating upslope. Details on this topic can not be presented in the frame of this overview; however, some relevant information regarding species, biodiversity and climate change can be extracted

from the following publications and the many cited references therein: Hughes 2000; Sala et al. 2000; Walther et al., 2002; Parmesan and Yohe 2003; Walther 2003; Parmesan 2006; Botkin et al. 2007; Fischlin et al. 2007; Hoegh-Guldberg et al. 2007, 2008; Kerr et al. 2007; Midgley et al. 2007; Thuiller 2007; Bonan 2008; Lee and Jetz 2008; Rosenzweig et al. 2008; Thuiller et al. 2008.

The science of climate change is touching upon a huge number of topics with many facets, which can not be fully accounted for here, and the selected aspects can not be elaborated in depth. Hence, an attempt has been made in the following sections to provide an extended selection of relevant references for further study, many of them in easily attainable journals. In the subsequent sections and after a short summary of the principal scientific fundamentals, the story of the Earth's climate over the past is recapitulated with enhanced emphasis on the Holocene. The section on the present-day climate builds on observations based on direct measurements of climate parameters, mainly temperature and precipitation. Finally, the outlook into the future reports on modelling studies of global and regional climate change assessments based on scenario-driven simulations. The limitations of model predictions, especially with respect to regionalisation, will also be touched upon. Regarding present and future climate, the results of the latest assessment report (AR4) published by the Intergovernmental Panel on Climate Change (IPCC 2007a) are used as a baseline. As the IPCC AR4 considers only published scientific papers up to 2005, for some aspects more recent work has been considered, in addition.

## 2 Scientific Basis

### 2.1 *The Climate System and Relevant Processes*

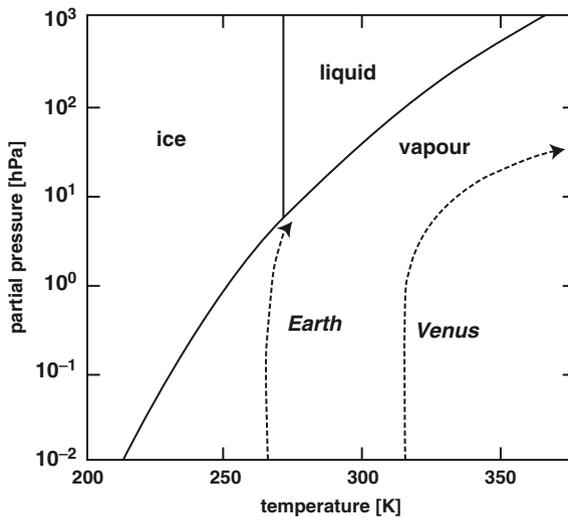
The usual definition of climate is that it encompasses the slowly varying aspects of the atmosphere–hydrosphere–land surface system. In some sense, climate is the average condition of the weather over several years to tens of years (averaging times need to be carefully chosen), as exemplified by the parameters viz., temperature, wind velocity, relative humidity, cloudiness and the amount of precipitation. Modern climate definitions include higher order statistics beyond mean values, such as the magnitudes of day-to-day or year-to-year variations, standard deviations or measures of shapes of parameter distributions.

Climate depends not only on atmospheric processes and composition, but also physical, chemical, and biological processes involving other components of the Earth system play a crucial role. In order to understand what the factors are which control the evolution of climate, the interactions among the different components of the Earth system need to be assessed (e.g. Brasseur et al. 1999). This adds to the complexity of the topic since the evolution and feedbacks involved run on a variety of different time scales. The atmosphere, the hydrosphere, the biosphere,

the cryosphere and the lithosphere are the five different Earth system regimes with widely varying impacts and time scales which make up the climate system.

The *abundance of water* in its three states of aggregation and a functioning global water cycle is of utmost importance for the climate system (e.g. Pagano and Sorooshian 2006; Quante and Matthias 2006). The phase of water depends on the temperature and pressure it is exposed to. At the normal range of atmospheric pressures and temperatures on Earth water can exist in all three of its basic states, as is evident from its phase diagram in Fig. 1, which shows the phase transition curves as a function of temperature and partial pressure (see Webster 1994). The Earth’s trajectory in Fig. 1, driven by an increasing water vapour greenhouse effect, intercepts the phase curves in the vicinity of the triple point of water (273.16 K), allowing the formation of a complex hydrological cycle. In contrast, because Venus is a star with a considerably warmer primitive surface temperature, the curve for Venus does not intercept any of the water phase transition lines at all. Water stays in the gaseous phase on this planet. The state curve for Mars starts at a relatively low temperature (~240 K; not shown here) and rapidly intercepts the vapour-ice phase transition.

All subcomponents of the climate system are involved in or maintain processes which can have a huge impact on climate. The interplay between radiation and convection in the atmosphere regulates the temperature at the Earth’s surface. The oceans, which cover about 72% of the surface area of our planet, influence climate by their large thermal inertia and their important role in taking up carbon dioxide from the atmosphere. If present, the cryosphere with extensive snow and



**Fig. 1** Phase diagram of water illustrating the possible occurrence of the three states of water for the range of temperatures and partial pressures observed at the Earth’s surface and in the lower atmosphere; ~190 K–325 K and 0–50 hPa partial pressure range at the surface. Figure adapted from Quante and Matthias (2006)

ice covered areas has a strong influence on the planetary albedo (Parkinson 2006). Besides their large influence on the Earth's albedo with a corresponding net cooling effect, the clouds are also contributing to a warming of the surface by absorbing infrared radiation and emitting it partly back towards the ground (e.g. Quante 2004). Clouds cover at any given time between 60 and 70% of the globe. Living organisms on land and in the oceans are involved in liberating oxygen and sequestering carbon in the Earth's crust and thereby reducing the CO<sub>2</sub> concentration of the atmosphere. The evolution of vegetation is strongly coupled with that of soil and climate, and there is a myriad of interactions involved (e.g. Berry et al. 2005; Barth et al. 2005). Plate tectonics exerts an influence on climate on time scales of more than millions of years through continental drift, creation of mountains (Turcotte and Schubert 2002) and volcanism (Robock 2000). Of all the biogeochemical cycles, the hydrological (e.g. Quante and Matthias 2006; Oki and Kanae 2006) and the carbon cycle (e.g. Houghton 2007; Doney and Schimel 2007) are the most relevant for climate and its evolution.

The major external forcing of the climate system comes from the sun. Everything on Earth relies on a steady energy flow provided by our central star. The amount of radiation produced by the sun is not constant, especially in the short, ultraviolet wavelengths. Due to changes in the magnetic structure of the gaseous sun, the solar activity shows variations, which are manifested in an 11-year cycle. Although attempts have been made, a firm theoretical coupling of this short-term solar activity fluctuations with climate changes could not be found. A slightly enhanced energy deposition in the stratosphere is among the most recognized effects. Some evidence for the influence of solar activity variations on the lower atmosphere and climate is critically assessed by Bard and Frank (2006), Foukal et al. (2006) and Haigh (2007). These variations play some role in the discussion on modern global warming, since if climate changes due to the sun were significantly large, it would be more difficult to extract the anthropogenic signal from the climate record (see Sect. 4.4). The story is different, however, when dealing with the long-term evolution of the sun; in its infancy, the sun's intensity was about 30% less than what is observed today (Gough 1981), the relatively moderate climate under these conditions is generally referred to as "faint young sun paradox" (e.g. Sagan and Chyba 1997). A further possible external forcing of Earth's climate might come via galactic cosmic rays and their influence on clouds (Marsh and Svensmark 2000; Carlsaw et al. 2002; Kristjánsson et al. 2004; Kirkby 2008). The related science and the potential magnitude of postulated effects is currently being debated and planned experiments at the Conseil Européen pour la Recherche Nucléaire (CERN) should, at least, provide some insight into underlying cloud microphysical processes.

In summary, Fig. 2 sketches the Earth system and its interactions, encompassing the physical climate system, biogeochemical cycles, external forcing, and the effects of human activities. For a more rigorous treatment of the different aspects concerning the climate system and underlying processes, the books by Peixoto and Oort 1992; Graedel and Crutzen 1993; Hartmann 1994; Brasseur et al. 1999; Seinfeld and Pandis 2006; Ruddiman 2008; and Pierrehumbert 2009 are recommended.

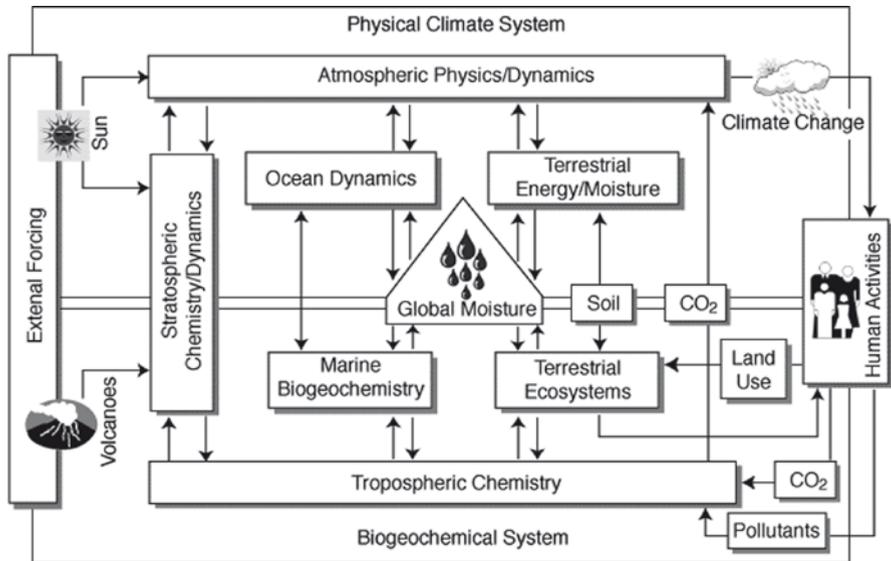
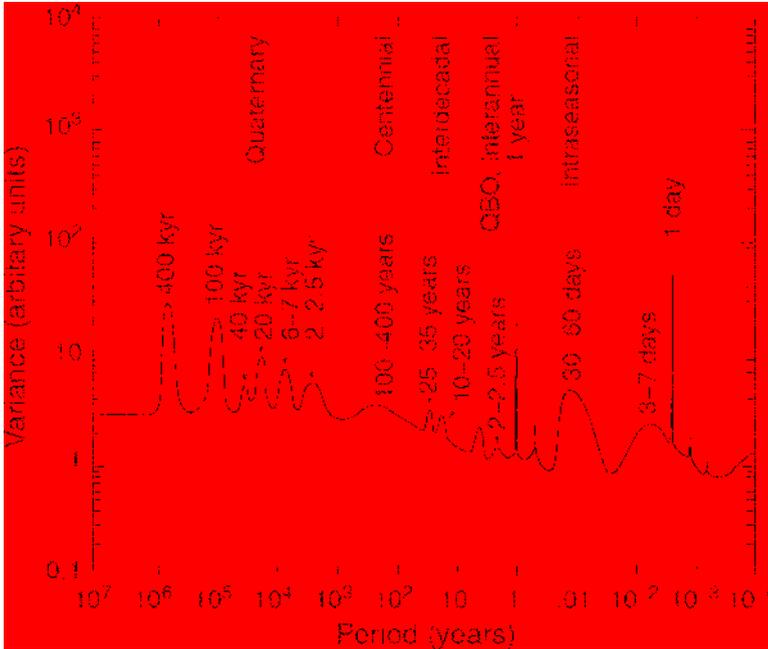


Fig. 2 Schematic diagram of the climate system and its interactions among different components of the Earth system (from Earth System Science: Overview, NASA)

## 2.2 Climate Variability

Climate varies on all time scales longer than its definition limit and on a wide range of spatial scales, from regional to global. The variability of climate can be expressed in terms of two basic modes: the forced mode with variations which are the response of the climate system to changes in external forcing, which themselves are not influenced by the climatic variables themselves, and the free mode variations due to internal instabilities and feedbacks leading to non-linear interactions among the various compounds of the climate system (Peixoto and Oort 1992; Ghil 2002a). Also, stochastic forcings could produce sudden impulses to the climate variables (Hasselmann 1976). It is due to this complex nature of the variability that a reliable identification of an anthropogenic influence on climate and especially its quantification turns out to be so difficult.

The temporal variability of the climate system over the last 10 million years can be visualized by reproducing a power spectrum for temperature near the surface (Fig. 3), which is a composite of several climatic time series. The sharp lines in the spectrum correspond to periodically forced variations (daily and yearly cycles), broader peaks arise from internal modes of variability, and the continuous segments of the spectrum reflect stochastically forced variations, as well as deterministic chaos resulting from the interplay of non-linear feedbacks (Ghil 2002b).



**Fig. 3** Composite power spectrum of climate variability over the last 10 Myr (after Ghil 2002b)

The signature immediately to the left of the peak at the 1-year-period reflects the influence of an interannual variability, which is known as El Niño-Southern Oscillation (ENSO) (Neelin et al. 1998) and involves internal modes of the tropical Pacific ocean-atmosphere variability. Periods of several decades as they occur in the spectrum are the subject of ongoing research. As the interdecadal variability is related to climate system internal processes this part of the spectrum might hold some clues for the extension of the predictability of the ocean-atmosphere system.

The spectrum part with periods between 1,000 years (1 kyr) to about 1 million years (1 Myr) in Fig. 3 represents the paleoclimatic variability. The temperature data underlying the spectrum far outside the instrumental record come from proxy indicators such as coral reef records, tree rings, marine sediment cores, and ice core records. The time span captured by the spectrum is called *ice age era* with its main glaciation cycles (Imbrie and Imbrie 1979), it occupies no more than about one-tenth of the documented Earth history. The past 2.5 Myr with the marked climatic swings are called Quaternary period.

The external forcings comprise variations caused by smaller fluctuations in the sunlight reaching the Earth, which occur over longer timescales. These involve changes in the shape of the Earth's orbit around the sun and changes in the Earth-sun distance at the time of the equinoxes. Early in the twentieth century, in Belgrade, Milutin Milankovic' produced a mathematical theory that became the basis for a general

explanation of the dynamical behaviour of the climate system, i.e. the major changes in past and future (Berger 1988). In particular, the theory which builds on deterministic astronomical variations in the orbital parameters of the Earth should explain the recent ice ages on Earth. The theory was not fully accepted by his contemporary scholars, and today we know that orbits are *not* everything (Broecker and Kunzig 2008), but basic orbit variability can be used to reconstruct climate records.

The prominent peaks around 20, 40 and 400 kyr in the spectrum in Fig. 3 are a result of quasi periodic variations in the Earth's orbit (Berger 1988), which affect the summer insolation at high latitudes. In intervals with relatively weak summer insolation snow deposited during winter does not melt completely, so over thousands of years thick ice sheets can be accumulated (enforced by the ice-albedo-feedback). The cycle of about 100,000 years is due to changes in the eccentricity of the orbit (ellipticity), variations of the tilt of the Earth's axis of rotation relative to the orbit plane (the obliquity) leads to the 41,000-year-cycle and the 23,000-year-(19,000 year) cycle marks the changing axial precession. Details of the orbital theory of climate variation are still under investigation (Paillard 2006).

Overall, it can be said that climate variability results from complex interactions of forced and free variations because the climate system is a highly non-linear, dissipative system with many sources of instabilities (Peixoto and Oort 1992). Feedback mechanisms act as internal controls of the system, they originate from special couplings between the open subcomponents and are of particular importance for the Earth's climate system. Feedbacks may either amplify or dampen the original distortions of the system. Important feedbacks are the water vapour-, cloud-, snow/ice-albedo-feedbacks, and the carbon-silicate cycle/carbon dioxide feedback (Held and Soden 2000; Stephens 2005; Kasting and Catling 2003).

### 2.3 Energy Balance and Greenhouse Effect

The Earth receives the overwhelming amount of its energy from the sun in the form of visible and near-infrared radiation; it is mainly this latter energy which warms its surface. Our planet is cooled by the emission of thermal infrared radiation into space. Treating the Earth as a blackbody radiator with an effective temperature  $T_e$ , the balance of incoming radiation and outgoing radiation leads to the following relationship (e.g. Wallace and Hobbs 2006):  $\sigma T_e^4 = S / 4(1 - A)$ , where  $\sigma$  is the Stefan-Boltzmann constant, where  $S$  is the solar flux at the distance Sun-Earth, and  $A$  is the planetary albedo. Using the appropriate numerical constants and quantities, solving the equation yields an effective emission temperature of 255 K. Reality is, however, slightly more complex, in that the Earth together with its atmosphere is *not* a perfect blackbody. The atmosphere warms the surface by the so-called greenhouse effect; parts of the infrared radiation emitted from the surface is selectively absorbed and re-emitted by infrared-active gases within the atmosphere. With the present-day incoming solar flux ( $\sim 1,368 \text{ Wm}^{-2}$ ), albedo ( $\sim 0.3$ ) and atmospheric

composition, a global mean surface temperature of 288 K results. The difference between the effective emission temperature and this surface temperature of 33 K is the magnitude of the actual greenhouse effect. A more complete treatment of the involved energy fluxes with emphasis on the energy budget resolved for additional processes is given in Kiehl and Trenberth (1997). The most difficult factor in the quantification of the energy fluxes through the atmosphere is the planetary albedo, which is determined by ocean and land surface characteristics (soil type, soil moisture, vegetation) and the three-dimensional cloud distribution in the atmosphere, which is responsible for the largest fraction. The relevant cloud (and aerosol) properties are difficult to predict. Thus, calculations of past or future climates based on energy principles (radiative and turbulent fluxes) are subject to high uncertainties, i.e. since clouds are involved in several feedback loops (Stephens 2005).

In the present-day atmosphere the most important greenhouse gases are water vapour and carbon dioxide, of which the former contributes about two-thirds of the associated warming. Lesser contributions come from methane, nitrous oxide, ozone and various chlorofluorocarbons. It is important to distinguish between long-lived greenhouse gases, which are removed slowly from the atmosphere on a time scale of hundreds to thousands of years, and short-lived greenhouse gases, which are removed within weeks to a year by condensation or fast chemical reactions. The short-lived greenhouse gases act primarily as a feedback mechanism. In the eighteenth century, the atmospheric concentration of most of these gases (with the exception of water vapour) began to be significantly altered due to emissions from power plants, industry, agriculture and animal farming as well as the mobility sector. The ongoing greenhouse effect discussion is driven by the anthropogenic emissions of  $\text{CO}_2$ ,  $\text{CH}_4$ ,  $\text{N}_2\text{O}$ ,  $\text{O}_3$  and CFC which show strong increases, especially over the last decades. It has to be noted that all of these gases have a relatively long residence time in the atmosphere and therefore, a high greenhouse warming potential.

Water vapour as the major player in the Earth's energy budget is buffered by the huge oceans on a time scale of a few weeks. This gas adjusts its atmospheric concentration in response to climate changes, and it has a strong positive feedback in the climate system, thus amplifying global warming caused by other forcings (Held and Soden 2000). Water vapour, although an important greenhouse gas, is not a prime driver of modern climate change (it plays an essential role via its positive feedback). As there are no significant anthropogenic emissions, water vapour has not become the subject of political regulatory protocols.

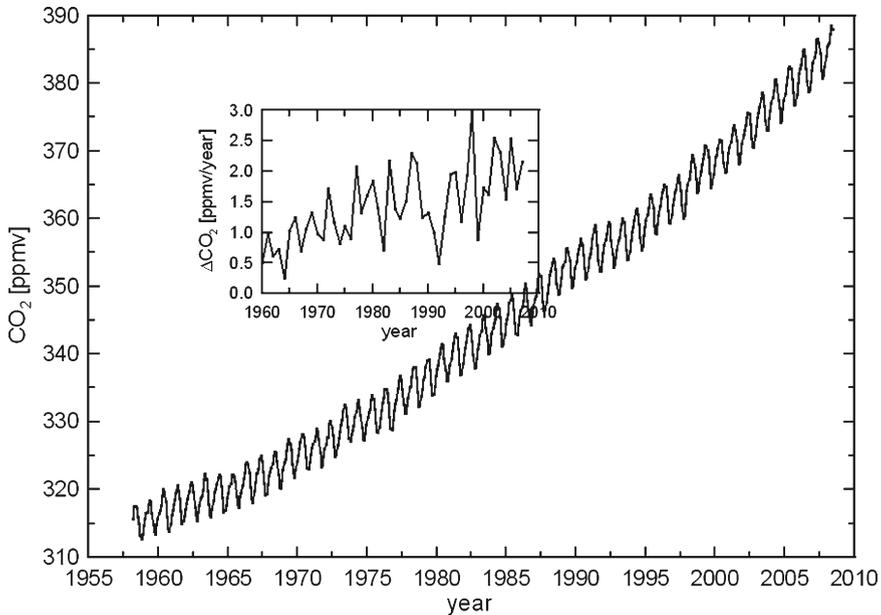
Of course human beings started influencing the atmospheric composition, and greenhouse gas concentrations, well before the massive industrialisation began. About 11,000 years ago stone-age farmers may have already altered Earth's climate by clearing forests and irrigating fields to grow crops. Besides resulting in changes to the albedo, these activities may have led to considerable amounts of  $\text{CO}_2$  and  $\text{CH}_4$  being emitted. It should be mentioned that some scholars have even put forward the hypothesis that this early interference with the climate system could possibly have averted the start of a new ice age (Ruddiman 2003, 2005). This controversial hypothesis is discussed, for example, by Claussen et al. (2005), since

the concentrations of CO<sub>2</sub> during the Holocene could also be attributed to natural processes (e.g. Broecker and Stocker 2006; Steffen et al. 2007).

## 2.4 CO<sub>2</sub>: The Flagship of Long-Lived Greenhouse Gases

CO<sub>2</sub> is currently the most important greenhouse gas (IPCC 2007a,b). It is involved in a complex carbon cycle (which also includes CH<sub>4</sub>), which in turn is closely connected via feedbacks to the total climate system (Houghton 2007). CO<sub>2</sub> is a trace gas on Earth and it is present in the atmosphere in a relatively low concentration. However, this concentration has varied strongly over geological time (Doney and Schimel 2007). CO<sub>2</sub> is implicated in virtually all of the great climate shifts in the history of our planet, including the coming and going of ice ages, warm ice-free states and the collapse of the Earth into a globally frozen state some 600 million years ago, the latter event being known as *snowball earth* (Hoffman and Schrag 2002; Pierrehumbert 2005). It was also involved in preserving conditions favourable to life on the young Earth, when the sun was much fainter (25–30%) than it is today (Kasting and Catling 2003). Of course, there is a lot to say about CO<sub>2</sub> in the biogeochemical carbon cycle and its many relations to climate. For further study, the overview publications compiled by Doney and Schimel (2007), Hansen et al. (2007a), and Houghton (2007) and the articles cited by those authors can be referred to.

Immediately before the beginning of the industrial era, the atmospheric CO<sub>2</sub> concentration amounted to about 280 ppm and varied between 260 and 280 ppm in the 1,000 of years previously (Houghton 2007). Because there is relatively little CO<sub>2</sub> in the atmosphere to begin with, modern human activity has the prospect of doubling its concentration within the twenty-first century. A large amount of the emitted CO<sub>2</sub> is taken up by the oceans and the vegetation, but a significant part remains within the atmosphere. State-of-the-art assessments based in part on the oxygen measurement methods developed by Ralph Keeling, the son of famous Dave Keeling, allocate about 35% of the emitted CO<sub>2</sub> to an ocean uptake and about 15% to land sinks, mainly the large forests distributed over the continents (Sabine et al. 2004; Broecker and Kunzig 2008). Due to its long atmospheric residence time, CO<sub>2</sub> is well mixed over the hemispheres and its concentration increase over time can be assessed to first order by determining the relevant values at one location. Today, there are approximately 100 stations worldwide where weekly air samples are collected and analysed for CO<sub>2</sub>. Keeling started the first systematic monitoring of CO<sub>2</sub> concentrations in 1958 at the Mauna Loa observatory, Hawaii, in the middle of the Pacific and far away from any large industrial sources (Keeling et al. 1976). The resulting concentration curve has become a sort of an icon of global warming in modern times, and is also known as *Keeling-curve* (Broecker and Kunzig 2008). In Fig. 4, the Mauna Loa CO<sub>2</sub> concentration is plotted, beginning in 1958 and including the most recent observed data. Atmospheric CO<sub>2</sub> rose from about 315 ppm when the measurements started to about 385 ppm by 2007, about



**Fig. 4** Monthly mean carbon dioxide concentrations measured at Mauna Loa Observatory, Hawaii. The small inlay graph shows annual mean carbon dioxide growth rates for the Mauna Loa station. Data provided by Dr. Pieter Tans, NOAA/ESRL

38% above the pre-industrial value of 280 ppm. The present concentration is the highest, which has been recorded over the last 800,000 years (Lüthi et al. 2008) and is probably also the highest over the last 20 million years (Pearson and Palmer 2000). The annual fluctuations seen on the overall rising curve are due to seasonally changes in the uptake of  $\text{CO}_2$  by vegetation mainly in the Northern hemisphere; the corresponding curve (not shown here) taken at the South Pole looks rather smooth in comparison. The inlay graph in Fig. 4 shows the  $\text{CO}_2$  increase rate at Mauna Loa, with positive values throughout and a clear tendency for higher values in later years. The subtle variations in the annual increase rates indicate the response of terrestrial and marine processes to climate variability (Doney and Schimel 2007). The increase rate in the global average concentration of atmospheric  $\text{CO}_2$  between 2000 and 2006 was  $1.93 \text{ ppm year}^{-1}$ . This rate is the highest since the beginning of continuous monitoring in 1958 and is a significant increase over the increase rates in earlier decades; the average growth rates for the 1980s and the 1990s were 1.58 and  $1.49 \text{ ppm year}^{-1}$ , respectively (Canadell et al. 2007). The authors also estimate that 35% of the increase in the atmospheric  $\text{CO}_2$  growth rate between 1970–1999 and 2000–2006 was caused by a decrease in the efficiency of the land and ocean sinks in removing anthropogenic  $\text{CO}_2$  (18%) and by an increase in carbon intensity of the global economy (17%). The remaining 65% was due to the increase in the global economy. Due to the still strongly increasing  $\text{CO}_2$  emissions, the concentra-

tion of CO<sub>2</sub> will continue to rise in the coming decades, thus further enhancing the anthropogenic greenhouse effect. In relation to these prospects and for the treatment of other greenhouse gases, Section 4 and the most recent UN climate assessment report (IPCC 2007a,b) should be referred to.

### 3 The Past: As Seen Through Proxies

This view on the climate of the past, is in its kernel orientated along the condensed treatments offered by Graedel and Crutzen (1993), Wallace and Hobbs (2006), and Pierrehumbert (2009) and complemented by selected new findings and some addendums.

#### 3.1 *Deep Past: The Geological Record*

During the first 700–800 million years of its history, the Earth was still under continual bombardement by planetesimals. Geologists call this early phase the *Hadean Epoch* (4.6–3.8 Gyr ago), for which the geological record is almost non-existent. Such impacts should have liberated water vapour and other volatile substances, thus resulting in the formation of a primordial atmosphere. Not surprisingly, the climate of the Hadean is very poorly understood, with only conceptual and model-based statements predominating (Kasting and Ono 2006).

A sparse geological record is available for the subsequent Archaean epoch (3.8–2.5 Gyr ago), and where Earth has become stable enough to allow for early life to develop in the oceans. The climate during the early Archaean was probably hot, as surface temperatures of  $70 \pm 15^\circ\text{C}$ , reconstructed from isotopic ratios of mountain land in South Africa, suggest (Kasting and Ono 2006). From paleo-weathering analyses it can be concluded, that during the late Archaean moderate surface temperatures must have predominated (Holland 1984). Although there are large uncertainties here, the balance of evidence suggests a relatively cool early Earth with respect to potential ocean vapourizing events (Kasting and Ono 2006).

##### 3.1.1 Faint Young Sun

It is known that the emission of radiation from stars increases gradually over their lifetime. The luminosity of the sun is believed to have increased more or less linearly by about 30% since the formation of the solar system (Gough 1981). This, in turn, means the solar radiation received by early Earth was considerably lower as compared to the present day value of  $1,370 \text{ W m}^{-2}$  at the top of the atmosphere. Assessments of historical climate must account for this less energetic start and which is known as faint young sun problem (Kasting et al. 1988).

In spite of the faint young sun, geological evidence indicates that the oceans have been largely free of ice and that the Earth's surface was not perpetually frozen during its early phase of evolution. Furthermore, there is also no convincing evidence in the geological record, which suggests that widespread glaciations occurred before, about 2.3 Gyr ago. The atmosphere must have contained much higher concentrations of greenhouse gases compared to modern times. The response of the inorganic carbon cycle and the carbonate-silicate-cycle to changes in climate kept the  $\text{CO}_2$  level in the atmosphere at a sufficiently elevated level, in this context volcanism and weathering were relevant processes within the negative feedback process on climate (Berner 2004).

### 3.1.2 Methane

In recent years, it has become obvious that  $\text{CO}_2$  alone, could not have compensated for the remarkably reduced solar input. Due to a much lower oxygen concentration in the early atmosphere, methane could have had a considerably longer life span which, in addition to a possible early source for methane (methanogens), made  $\text{CH}_4$  a candidate for the greenhouse gas responsible for stabilizing the Earth's early climate. The details of the role of methane in early climate are quite complicated and only partly settled. For a more in-depth discussion on methane the reader is referred to Kasting and Catling (2003), Kasting (2005), and Kasting and Ono (2006).

### 3.1.3 Snowball Earth

Acting in concert with orbit-steered periodic patterns of solar radiation, both the ebbing and rising oceans as well as the forming continents lead to changes in the Earth's reflectivity at the surface (albedo). Land areas can also influence the albedo considerably by the formation of ice caps and by vegetation growth. The world-wide distribution of types, conditions, and position of rocks permit a determination of the periods and extent of snow and ice appearance on the globe (Broecker and Kunzig 2008). This evidence leads to the conclusion that the Earth has passed through a number of glaciation cycles. The earliest verifiable extensive glacial epoch falls in the time interval 2.4–2.2 Gyr BP (Palaeoproterozoic glaciations). Major glaciations extending all the way into the tropics have been termed "snowball Earth" (Hoffman and Schrag 2002; Pierrehumbert 2002; Allen 2006). "Hard snowball Earth" events are characterized by global sea ice cover (Pierrehumbert 2005). The second extended glaciation period with at least two events occurred between 750 and 600 Myr ago and the third, the Permian glaciation about 280 Myr ago. The ice-albedo feedback mechanism is involved in building-up the snowball, whereas an increasing greenhouse gas effect, carbonate formation and windblown dust events are among the complex processes involved in reversing this phenomenon, with possible abrupt flips into an ice-free state (extended hothouse phase). The

role of CO<sub>2</sub> during the Phanerozoic eon is explicitly treated by Crowley (2000b) and an excellent review on its paleo-proxies is given by Royer et al. (2001).

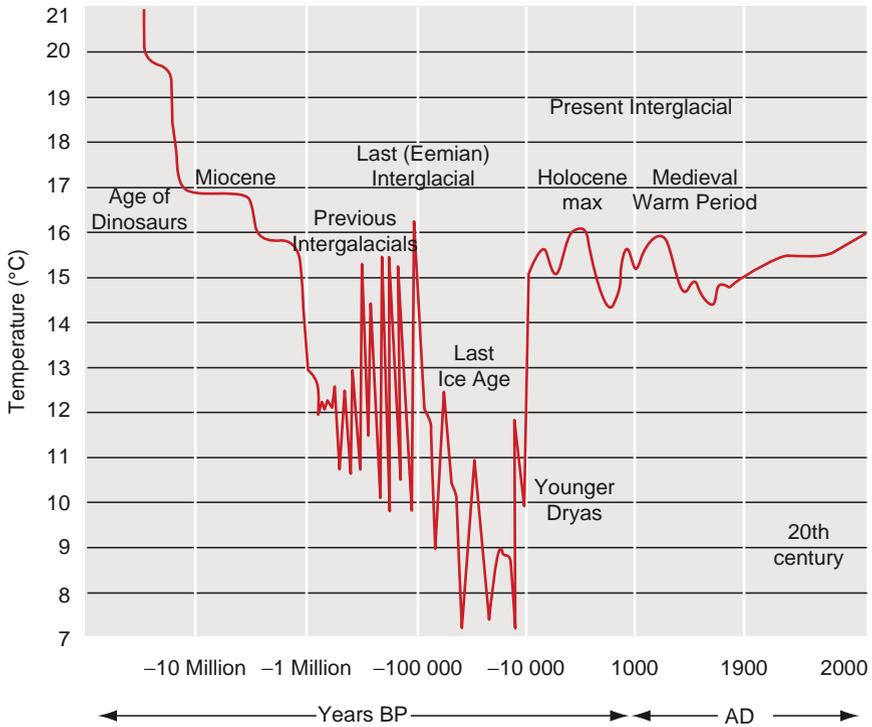
### ***3.2 The Past 100 Million Years***

During the Cretaceous epoch, which ended 65 Myr BP, surface air temperatures were higher than they are at present. This is especially true for higher latitudes. An extended interval of cooling followed the Cretaceous epoch, which went along with declining CO<sub>2</sub> concentrations. The cooling culminated in the Pleistocene glaciation, which began about 2.5 Myr ago. Plate tectonics regulating the carbonate-silicate cycle along with the glaciation of the Arctic continent around 30–15 Myr ago and its drift toward higher latitudes are believed to have been responsible for this gradual trend in cooling. Since about 65 million years ago, Earth's climate has undergone a significant and complex evolution. Gradual shifts driven by tectonic processes and periodic cycles caused by orbital rhythms are responsible for rapid shifts and extreme transitions. An example is the Late Paleocene Thermal Maximum at about 55 Myr ago, which dominates the Cenozoic era (Zachos et al. 2001). This event could be the best ancient climate analogue for future increases in atmospheric CO<sub>2</sub>, and although there has been much progress in recent years in revealing the “carbon mysteries” (Pagani et al. 2006) there are still many aspects which remain unknown. The sudden global warming around 55 Myr ago provides evidence for high climate sensitivity to atmospheric CO<sub>2</sub>. The fast changes recorded in deep-sea sediment isotope cores have especially helped to improve our perspective on the mechanisms involved in rapid alterations in the climate system. A detailed treatment of the geological evidence and paleogeography can be found in Pierrehumbert (2009). The Cretaceous hothouse climate and the Pleistocene icehouse climate represent opposite extremes of the Earth's typical climate state over the past 500 million years.

### ***3.3 The Past Million Years***

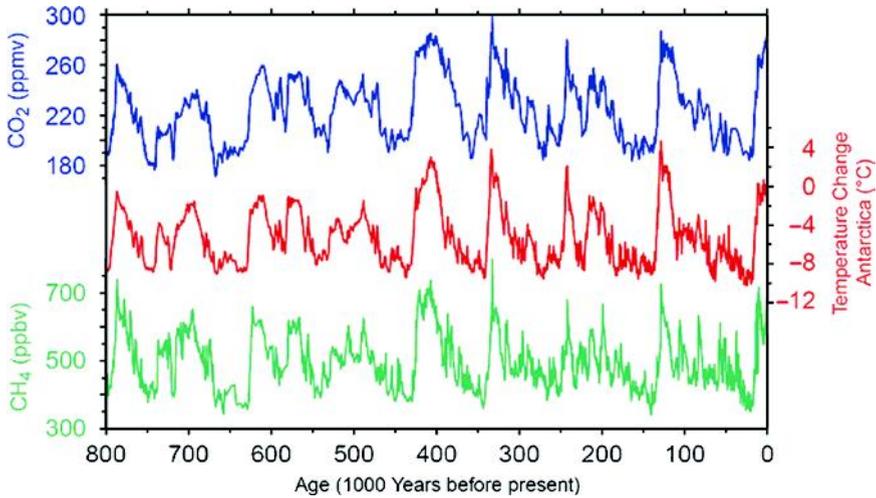
In the past 2.5 million years, the so-called Quaternary period, the Earth's climate has been marked by temperature swings between extended glacial periods, which were characterized by thick ice sheets covering large parts of North America, Northern Europe and Siberia, and interglacial times characterised by an ice covering only in Antarctica and sometimes Greenland, as is the case today. A schematic illustrating these glacial and interglacial swings and putting the temperature trends of this period in an historical perspective is displayed in Fig. 5.

Drilling is a valuable way of gaining information about the Pleistocene period. Sediment cores reaching a few million years back in time and ice core records dating back to about 800,000 years have been extensively analysed. The ice at the base of the Antarctic is about one million years old. The composition of the past air is



**Fig. 5** Temperature trends for the last 10 Million years. Note the different scalings on the time axis: the twentieth century is shown in linear scale. Earlier periods are shown in terms of increasing powers of ten but are linear within each period (figure adapted from Bureau of Meteorology, Australia)

preserved in bubbles within the ice and stable isotopes, derived from the ice itself, are essentially recorders of the temperature at that time. Changes in the atmospheric  $\text{CO}_2$  concentrations over the past 800 kyr, are illustrated as a composite of EPICA Dome C, Vostok and Taylor Dome ice cores together with the EPICA Dome C temperature anomaly record in Fig. 6; this figure is taken from Lüthi et al. (2008). Similar data for methane can be found in Louergue et al. (2008). Based on the analysis of ice records like the ones shown in Fig. 6 and an alignment with many marine sediment cores, it is clear that on time scales of tens of thousands of years or longer, temperature and some other climate parameters vary coherently with one another and that the variations are global in extent. Atmospheric  $\text{CO}_2$  and methane concentrations have risen and fallen synchronously with temperature. The correlation between  $\text{CO}_2$  and temperature does not, however, fully determine the underlying causes and which as detailed time assessments reveal (Pierrehumbert 2009), for many parts of the record  $\text{CO}_2$  is leading temperature by a few hundred years, thereby suggesting that there are different mechanisms acting as compared to those dominant in the present-day anthropogenic greenhouse warming. Over the last



**Fig. 6** Composite  $\text{CO}_2$  (upper curve) and  $\text{CH}_4$  (lower curve) concentrations from EPICA Dome C, Vostok and Taylor Dome ice cores cover the past 800 kyr. (from Lüthi et al. 2008 and Loulergue et al. 2008). The temperature anomaly (middle curve) is the deviation from the mean temperature of the last millennium (Jouzel et al. 2007)

800,000 years, the Milankowitch cycles were the dominant causes and pace-makers for climate variability. Global temperatures cooled at irregular rates during the extended glacial epochs and rose much faster at the beginning of the interglacials. These interglacials recurred during the recorded period at intervals of roughly 100,000 years and had durations typically of about 15,000–20,000 years. The last glacial maximum occurred around 20,000 years ago. The pronounced climatic swings during this Quaternary period are believed to be driven by subtle variations in the orbit parameters, which affect the summer insolation at high Northern latitudes. When the summer insolation was relatively weak for longer periods, the winter snow could partially survive, leading to thick ice sheets over a time span of thousands of years, with the ice-albedo effect amplifying the orbital forcing. Although its correlation with temperature is not totally resolved, because  $\text{CO}_2$  is a greenhouse gas, it is fairly certain that a rise in  $\text{CO}_2$  concentration warms the planet and reinforces the termination of ice ages, whereas a decrease in  $\text{CO}_2$  concentration enhances cooling and reinforces the onset of glaciation.

The data shown in Fig. 6 are the longest and most carefully studied records of polar-ice climate histories published. The record tells us that the highest temperature occurred during this period around 130 kyr ago. The  $\text{CO}_2$  concentration during the past 800 kyr never exceeded the present-day value of 385 ppm. For the many interesting details, such as short-term fluctuations as disclosed by the analysis of the sediment and ice core records, the articles by Jouzel et al. (2007), Lüthi et al. (2008), Loulergue et al. (2008) and the book by Pierrehumbert (2009) should be consulted. Interesting findings concerning the important Asian monsoon over the past quarter million years are reported by Wang et al. (2008).

The oceans, which cover more than two-thirds of our blue planet, definitely contribute as strongly as the large ice sheets to the timing of climate events. These waters move in a global circulation system, which is driven by subtle density differences and thereby transport huge amounts of heat energy. Ocean circulation is thus an active and highly nonlinear player in the global climate game. Increasingly clear evidence implicates ocean circulation in abrupt and dramatic climate shifts, such as sudden temperature changes in Greenland on the order of 5–10°C and massive surges of icebergs into the North Atlantic Ocean – events that have occurred repeatedly during the last glacial cycle (Rahmstorf 2002).

### ***3.4 The End of the Last Ice Age and the Holocene***

The records of climate proxies for the past 20,000 years, i.e. from about the end of the last glacial maximum, are so complete that not only the typical temperature and precipitation patterns are known but also features such as the geographical ranges of specific species of trees.

The transition from the last glacial to the current interglacial epoch was dramatic. The ice sheets started shrinking around 15,000 years ago. By 12,000 years ago, the Laurentide ice sheet was pouring large volumes of melt water into newly formed lakes and rivers setting the stage for flood events which shaped the landscape, as it is known today.

One of the most striking abrupt change events occurred as the Earth came out of its most recent ice age, the result was a sudden strong warming, called the Bolling warm period (Pierrehumbert 2009), which brought the climate back to a full interglacial warm period. However, this event did not last long and the climate reverted back abruptly to full glacial temperatures. This relapse into ice age conditions lasted about 800 years and ended about 11,700 years ago and is referred to as the Younger Dryas. The timescale of the Younger Dryas event is much shorter than that of the orbital cycles. One explanation for this sudden event, which is still under discussion but favoured by leading scientists (Broecker and Kunzig 2008), could be the massive draining of a huge glacial lake into the ocean, thus influencing the deep water formation causing a shutdown of the oceanic thermohaline circulation and therefore, reducing the poleward heat transport by the Gulf Stream. Whether the Younger Dryas event was truly global in extent, such as the climatic swings on the time scales of 10,000 years and longer have been, is still under debate. Signatures of this event, however, show up clearly in the Greenland ice cores and in European proxy climate records.

Compared to these strong changes, the Bolling warm period and the Younger Dryas in the first 1,000 years after the end of the ice age and the relatively smooth changes over the past 10,000 years, the years of our civilisation, and a tranquil warmth and cessation of high amplitude millennial variability appear, from the climate dynamics point of view, to be quite boring. However, this will most probably not remain like this since human beings started to interfere intensively with the system.

### 3.4.1 Holocene

The Holocene is the youngest part of the Earth's history. Its definition is given geologically and corresponds to the post-glacial warm period, beginning with the glacier retreat from the moraines in central Scandinavia. Its start is dated as 11,550 years ago. The Holocene has not witnessed the large temperature swings which characterized the previous glacial epochs. There were a few smaller scale temperature variations, and some of these appear to have had considerable impacts on some societies which settled and started agriculture within this epoch. The Little Ice Age, starting in the fourteenth century and lasting into the nineteenth century, is one popular example for this new type of climate impact. A Viking colony in Greenland failed, the population of Iceland declined substantially and farms were abandoned in some parts of Norway and the Alps (Wallace and Hobbs 2006). A comprehensive treatment of the dynamics of natural climate variability during the Holocene based on an integrative analysis combining theoretical assessments, observational data and climate modelling is provided by Fischer et al. (2004). Possible anthropogenic influence on the early Holocene climate is discussed by Ruddiman (2003, 2005) and Claussen et al. (2005).

Reconstructed palaeoclimate variabilities are based on ice cores, lake sediments, tree rings, corals and marine sediments. During the Holocene, the global distribution of rainfall has varied quite remarkably. Due to the changing orientation of the Earth's axis in orbit around the sun and a decreasing Sun–Earth distance, by the 9 kyr BP the average solar radiation over the Northern hemisphere was 8% higher in July and the same percentage lower in January as compared to today. An enhanced temperature contrast between ocean and land was the major effect, causing stronger oceanic evaporation and stronger precipitation over the continents. The gradual formation of a strong monsoon pattern probably also resulted, which in turn led to increased vegetation. In a Northern centric view, the years with the warmest summers started about 7,000 years ago and are sometimes called *Climatic Optimum*. However, this was certainly not optimal for the continental areas in the Southern hemisphere, which experienced a weak cooling.

As fossils from lake beds indicate, areas of the Sahara Desert were vegetated, the so-called “green Sahara”, and some parts were even swampy around 6,000 years ago. Parts of the Middle East where the first agricultural settlements were formed have subsequently become extremely dry. In response to weakening insolation forcing, a reduced African monsoon and abrupt hydrological changes resulted in progressive drying of regional terrestrial ecosystems. There is an overall consensus that during the Holocene optimum, the Sahara was greener than what it is today (Claussen 2003).

Large scale dust mobilisation followed from around 4,300 years before the present. Today's deserts and regional meteorological systems were established at about 2,700 year BP (Kroepelin et al. 2008).

Variations on smaller timescales in the order of decades to a century were superimposed on longer term variability (Witt and Schumann 2005), and they also had their influence on humans relying on a suitable climate. The collapse of the Mayan

and Anasazi civilizations has been attributed to the inability of these societies to adapt to trends towards drier climates.

The warmed continental interiors in Northern latitudes created a climate which was comfortable for humans and was also, without a doubt, comfortable for living organisms in general. These conditions definitely contributed to the expansion of human activity in these latitudes and were a major determining factor in the history of civilization (Cloud 1978; Lamb 1995).

A comprehensive study based on selected proxy-based reconstructions of different climate variables, together with state-of-the-art time series of natural forcings (orbital variations, solar activity variations, large tropical volcanic eruptions, land cover and greenhouse gases), can be found in Wanner et al. (2008). Here, the most recent results concerning all aspects ranging from desertification to monsoon shifts to Alpine glacier advances are presented and discussed. The work is underpinned by results from General Circulation Models (GCMs) and Earth System Models of Intermediate Complexity (EMICs) to establish a most complete explanatory framework. A meandering tour of Holocene paleoclimate research focussing on Europe and Northern Africa is provided by Battarbee and Binney (2008), these authors bring together an interdisciplinary group of paleoclimatologists.

### 3.4.2 The Last Millennium: The Late Holocene

Detailed records of climate change during the last millennium come from proxy indicators such as mountain glaciers, tree rings, and corals stored in data archives. Additionally, historical observations recorded by humans over several centuries for several regions are also available.

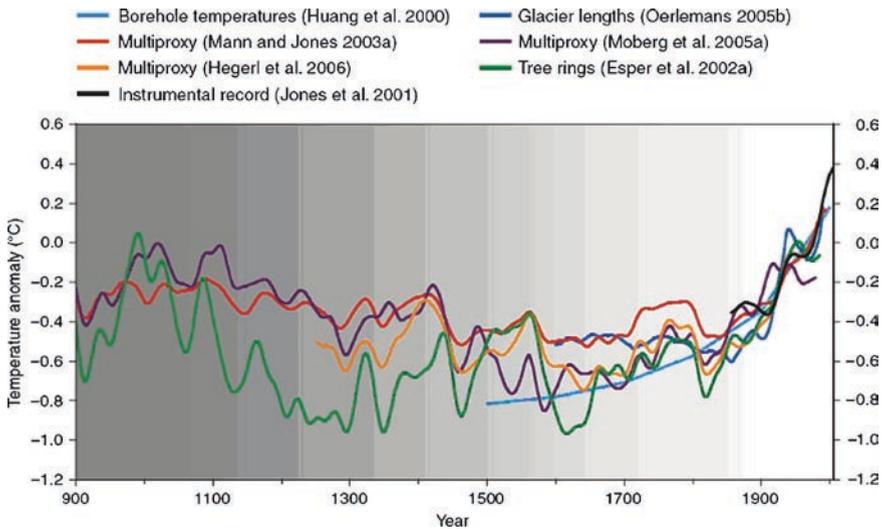
Up to the beginning of the last century climate changes were small and varied in pattern from region to region. A small cooling in high Northern latitudes surrounding the North Atlantic Ocean and known as the Little Ice Age is one of the climatic fluctuations of interest.

Several attempts have been made to synthesize high-resolution records from ice cores, glacier lengths, tree rings, corals and borehole temperatures into a single estimate of the Northern hemisphere or global temperature changes over the last millennium. These large-scale surface temperature reconstructions are not without problems. Proxy records are all linked in some way to temperature, and in addition, they are also affected in a complex way by other climatic, biological and ecological factors.

The first of these complex reconstructions were published in 1998 and 1999 by Mann, Bradley, and Hughes. The basic conclusion of Mann et al. (1998, 1999) was that the late twentieth century warmth in the Northern hemisphere was unprecedented during, at least, the last 1,000 years. This research received wide attention, in part because it was illustrated with a simple graphic, the so-called hockeystick curve, that many interpreted as definitive evidence of anthropogenic causes of recent climate change. The research was given prominence in the 2001 report of the Intergovernmental Panel on Climate Change. There is still an

ongoing debate about the details of some of the published temperature records, the means of their reconstruction and the underlying statistical measures. Some basic points of concern were addressed by von Storch et al. (2004) and McIntyre and McKittrick (2005). More recent updates or evaluations of the temperature reconstructions, methods and statistical aspects can be found in Mann and Jones 2003; Jones and Mann 2004; D’Arrigo et al. 2006; NRC 2006; Rybski et al. 2006; and Mann 2007.

Despite the aforementioned scientific debate it can be stated that large-scale surface temperature reconstructions yield a generally consistent picture of temperature trends during the preceding millennium, including the relatively warm conditions centred around 1,000 B.P. (identified by some as the “Medieval Warm Period”) and a relatively cold period (or “Little Ice Age”) centred around 1,700. The existence of a Little Ice Age from roughly 1,500–1,850 is supported by a wide variety of evidence including ice cores, tree rings, borehole temperatures, glacier length records, and historical documents (NRC 2006). Figure 7 shows a compilation of large-scale surface temperature reconstructions from different research groups, each using its own methodology and selection of proxies, as well as the instrumental record (beginning in 1856) of global mean surface temperature. The reconstructed temperatures all show a more or less pronounced gradual decline for almost 900 years after the medieval warm period and a considerable warming



**Fig. 7** Smoothed reconstructions of large-scale (Northern Hemisphere mean or global mean) surface temperature variations over the last 1,100 years from six different research teams are shown along with the instrumental record of global mean surface temperature. Each curve portrays a somewhat different history of temperature variations and is subject to a somewhat different set of uncertainties that generally increase going backward in time (as indicated by the *gray shading*). Figure adapted from NRC (2006)

which began in the middle of the nineteenth century. Comparing the results of the different methods reveals that there appears to be much more certainty in the reconstructed temperatures for the last 400 years.

The warming in the last century displayed in Fig. 7 stands out as *unusual* within the displayed temperature series.

The special assessment report of the National Research Council (NRC 2006) concludes that it can be said with a high level of confidence that global mean surface temperature was *higher* during the last few decades of the twentieth century than during any comparable period during the preceding four centuries. The NRC committee placed less confidence in large-scale surface temperature reconstructions for the period from A.D. 900 to 1600. Presently available proxy evidence indicates that temperatures at many, but not all, individual locations were higher during the past 25 years than during any period of comparable length since A.D. 900. Based on the analyses presented in the original papers by Mann et al. (1998, 1999) and newer supporting evidence, the NRC committee finds it plausible that the Northern hemisphere was warmer during the last few decades of the twentieth century than during any comparable period over the preceding millennium (NRC 2006). Reconstruction attempts for the past two millennia are presented in Mann and Jones (2003), Jones and Mann (2004), Mann (2007), and these proxy reconstructions suggest that the late twentieth century warmth is anomalous also in the context of the past 2,000 years.

Concluding a long-term persistence study on six of the reconstructed Northern hemisphere temperature records, Rybski et al. (2006) state: “Accordingly, the hypothesis that at least part of the recent warming cannot be solely related to natural factors, may be accepted with a very low risk, independently of the database used”. The unprecedented warming during the last 150 years will be discussed more closely in the next chapter.

## 4 The Anthropocene: The Instrumental Climate Record

Over the last two centuries, human activities have profoundly altered many aspects of the Earth’s system, including its climate and its biogeochemistry, and to the point where some argue that we are entering a new geological era, the Anthropocene (Crutzen and Stoermer 2000; Steffen et al. 2007). In other words, the atmospheric concentration of some greenhouse gases has been enhanced to such an extent (IPCC 2007a) during the course of the Anthropocene, that regional to global changes in surface temperature and other climate parameters are to be expected.

The instrumental era of meteorological observations began with the invention of the thermometer by Galileo in the late sixteenth century and the mercury barometer by Torricelli in the seventeenth century. Instrumental records are, by far, the most reliable of all climate data, as they are precisely dated and employ physical calibrations. The modern instrumental measurements of climate parameters include thermometer-based surface temperatures from land regions and from the oceans,

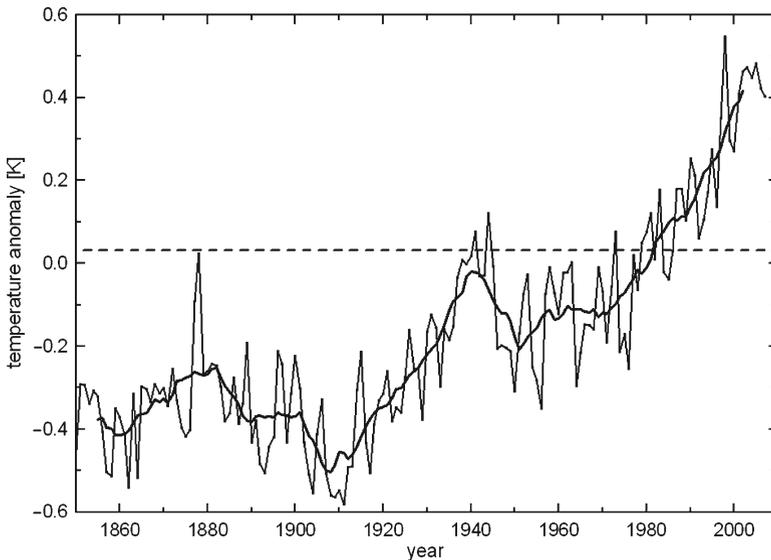
sea level pressure, continental and oceanic precipitation, sea ice extent, wind, and humidity. Unfortunately, it has only been since the late 1800s that enough stations have been systemically recording these data to permit reasonable estimates of global means.

There is a huge body of literature available addressing many aspects of climate change over the instrumental observation period. The most comprehensive assessment is published in the fourth assessment report of the IPCC (IPCC 2007a, Chap. 3). Only a few *key points* can be highlighted by this review.

## 4.1 Temperature Changes

Reliable global means of surface temperature are available since 1850. Several compilations by different research groups provide gridded monthly mean temperature estimates back through the mid nineteenth century on a large scale (Jones and Mann 2004). The reconstructions by the different groups are very similar, although there are some disagreements mainly in the early 1900s when station coverage was still sparse.

Figure 8 shows a time series of estimated annual global mean surface temperatures for the time period from 1850 to 2007 based on thermometer measurements; the values displayed in the figure are deviations from the mean of the base period 1961–1990. This time series is being compiled jointly by the Climatic Research



**Fig. 8** Global annual mean temperature anomalies (from the mean of the base period 1961–1990) for the years 1850–2007, the smoother line shows an 11-year running average. Data provided by Climatic Research Unit, Norwich, UK, and Hadley Centre, UK Met Office

Unit and the UK Met. Office Hadley Centre; see Brohan et al. (2006) for details and error estimates. There is little temperature trend between 1850 and 1920, but between 1920 and 2007 the global mean temperature has risen by about 0.8°C. This has not been a steady rise though, temperature rose quickly during the 1920s to early 1940s, stabilized or fell slightly from the 1940s through to the late 1970s, and has again risen since 1980 to its present value. The 1990s were the warmest complete decade in the series. The warmest year of the entire series was 1998, with a temperature of 0.546 K above the mean of the current climatic period (1961–1990). Twelve of the thirteen warmest years in the series have now occurred in the past 13 years (1995–2007). The 10 warmest years on record are 1998, 2005, 2003, 2002, 2004, 2006, 2001, 2007, 2001, and 1997. Increased concentrations of greenhouse gases in the atmosphere (see e.g. Fig. 4 for CO<sub>2</sub>) due to human activities are most likely the underlying cause of warming in the twentieth century (IPCC 2007a).

The warming of the Earth is not distributed evenly over the globe, there are quite a few regional and seasonal differences to report. Continents show more rapid temperature increases compared to the oceans. A distinct warming can be observed in winter and spring in higher Northern latitudes, i.e. in the Arctic. Average Arctic temperatures increased at almost twice the global average rate over the past 100 years.

Widespread changes in extreme temperatures have also been observed over the last 50 years. Cold days, cold nights and frost have become less frequent while hot days, hot nights, and heat waves have become more frequent (IPCC 2007a; Easterling et al. 2000a). Several studies have shown that anthropogenic warming trends in Europe imply an increased probability of very hot summers. For example, the summer of 2003 with its record-breaking central European summer temperatures was probably the hottest since 1,500 (Stott et al. 2004), and a shift towards a regime with an increased temperature variability in addition to increases in mean temperature may have been responsible for this remarkable heat wave (Schär et al. 2004).

In addition to the changes in surface temperatures with a general warming tendency, analyses of balloon-borne and satellite measurements show a cooling of the stratosphere, which is consistent within greenhouse gas-driven climatic change. The observed pattern of tropospheric warming and stratospheric cooling is *very likely* due to the combined influences of greenhouse gas increases and stratospheric ozone depletion.

## 4.2 Other Changes

### 4.2.1 Hydrological Parameters

Due to a high variability of the water cycle on all time scales, significant trends in hydrological quantities are difficult to substantiate. Nevertheless, Huntington (2006) concludes a review of available studies that although data are often incomplete in

spatial and temporal sense, the weight of evidence indicates an ongoing intensification of the water cycle. One of the central questions is, however, whether global warming leads to more evaporation and hence to an increased water vapour content in the atmosphere. Trenberth et al. (2005) observed increases in precipitable water over the global oceans since the mid-1980s. But caution is advised by Trenberth et al. (2005) for extracting information on trends in tropospheric water vapour from global reanalysis products, since they suffer from spurious variability and trends related to changing data quality and coverage. Satellite data presented by Soden et al. (2005) supports column-integrated moistening trends for the years from the mid-1980s. A most recently published evaluation of in situ surface air and dew point temperature data found very significantly increasing trends in global and Northern hemispheric specific humidity (Dai 2006). More details on evaporation changes are given in Quante and Matthias (2006).

The increased atmospheric moisture content associated with a warming might be expected to lead to increased global mean precipitation. However, precipitation is also strongly influenced by changes in the tropospheric energy budget and the atmospheric circulation, so spatio-temporal patterns in precipitation changes are likely to be complex. Concerning precipitation, the most recent IPCC reports states (IPCC 2007a): Global terrestrial annual mean precipitation showed a small upward trend over the twentieth century of approximately 2.1 mm per decade (based on the Global Historic Climatology Network, GHCN, data). Long-term trends from 1900 to 2005 have been observed in the amount of precipitation over many large regions. Significantly increased precipitation has been observed in Eastern parts of North and South America, Northern Europe and Northern and central Asia. The frequency of heavy precipitation events has increased since 1950 over most land areas, consistent with warming and observed increases of atmospheric water vapour. Drying has been observed in the Sahel, the Mediterranean, Southern Africa and parts of Southern Asia. Precipitation is highly variable spatially and temporally, and data are limited in some regions. More intense and longer droughts have been observed over wider areas since the 1970s, particularly in the tropics and subtropics. Increased drying linked with higher temperatures and decreased precipitation have contributed to changes in drought (IPCC 2007a).

#### 4.2.2 Cryogenic Components

The warming in the Arctic caused a decrease in snow cover since 1980, i.e. in spring time, as well as a decrease in sea ice of about 2.7% per decade, the latter is especially pronounced in late summer (September) showing a trend of 7.4% per decade. A record minimum has been observed in September 2007, which is not included in the reported trends. Also, in other parts of the globe changes in cryospheric components can be observed. Mountain glaciers and snow cover have declined on average in both hemispheres. Losses from the ice sheets of Greenland and Antarctica have very likely contributed to the observed sea level rise between 1993 and 2003 (IPCC 2007a). Flow speed has increased for some Greenland and

Antarctic outlet glaciers, which drain ice from the interior of the ice sheets. The corresponding increased ice sheet mass loss has often followed thinning, reduction or loss of ice shelves or loss of floating glacier tongues.

### 4.2.3 Sea Level Rise

Global average sea level rose at an average rate of  $1.8 \text{ mm year}^{-1}$  (range 1.3–2.3) over the years 1961–2003. The rate was faster over the years 1993–2003, with about  $3.1 \text{ mm year}^{-1}$  (range 2.4–3.8). Whether the faster rate for 1993–2003 reflects decadal variability or an increase in the longer-term trend is unclear. There is *high confidence* that the rate of observed sea level rise increased from the nineteenth to the twentieth century. The total twentieth century rise is estimated to be 0.17 m (range 0.12–0.22) (IPCC 2007a). An extended evaluation by Church, and White (2006) concluded a global mean sea-level rise from January 1870 to December 2004 of 0.195 m, a twentieth century rate of sea-level rise of  $1.7 \pm 0.3 \text{ mm year}^{-1}$  and a significant acceleration of sea-level rise of  $0.013 \pm 0.006 \text{ mm year}^{-2}$ .

### 4.2.4 Wind

Mid-latitude westerly winds have strengthened in both hemispheres since the 1960s. There is observational evidence for an increase in intense tropical cyclone activity in the North Atlantic since about 1970, correlated with increases of tropical sea surface temperatures (IPCC 2007a). There are also suggestions of increased intense tropical cyclone activity in some other regions (Emanuel 2005; Webster et al. 2005) where concerns over data quality are greater. Multi-decadal variability and the quality of the tropical cyclone records prior to routine satellite observations in about 1970 complicate the detection of long-term trends in tropical cyclone activity. There is no clear trend in the annual numbers of tropical cyclones.

## 4.3 Attribution

A central topic within the climate debate of the recent years was whether, or to what an extent, the observed changes in climate can be attributed to anthropogenic emission of greenhouse gases or are due to natural variability. This extensively conducted discussion can not be fully summarized here; however, some aspects could be emphasized.

One central argument of the so-called “climate sceptics” is based on observed changes in the sun’s activity and a rise in solar radiation intensity. The question arises: to what extent are changes in the sun responsible for the recent warming? Although the rarity of the current episode of high average sunspot numbers (Solanki et al. 2004) may indicate that the sun has contributed to the unusual climate change

during the twentieth century, the work of Solanki and Krivova (2003) reveals that solar variability is unlikely to have been the dominant cause of the strong warming observed during the past three decades. A model-based exhaustive study by Allen et al. (2006) concludes that increasing *anthropogenic gas concentrations* produced a 0.3–0.5 K per century warming from 1906 to 1996 and are the dominant cause of global warming after 1976. In contrast, Scafetta and West (2006, 2007) report that empirical analyses suggest that *solar variability* accounts for as much as 69% of the twentieth century warming and 25 to 35% of recent warming, globally. The IPCC report (AR4) compares observed continental- and global-scale changes in surface temperature with results simulated by climate models using natural and anthropogenic forcings. One relevant conclusion is (IPCC 2007a): “The observed widespread warming of the atmosphere and ocean, together with ice mass loss, support the conclusion that it is *extremely unlikely* that global climate change of the past fifty years can be explained without external forcing, and *very likely* that it is not due to known natural causes alone”.

A multivariate analysis using the most reliable estimates of the relevant parameters together with the observed surface temperature record from 1889 to 2006 enabled Lean and Rind (2008) to assess the global and regional responses to different forcings. Their conclusion with respect to the solar influence is that solar forcing contributed *negligibly* to long-term warming over the past 25 years and about 10% of the warming over the last century. This result is in strong contrast to the 69% estimate of Scafetta and West (2006).

## 5 The Future: The “Era” of Model Assessments

The only rational way to attain reasonable estimates of future climate change is by the use of complex physical models of the climate system. In order to produce climate projections for decades or centuries to come, reasonable anthropogenic greenhouse gas and aerosol emission data as well as land use scenarios are needed in conjunction with sophisticated computer codes as forcing boundary values. Climate models and emission scenarios are briefly introduced in the following sections before the results from such model assessments of future climate situations are presented.

### 5.1 *Climate Models*

Climate models are based on mathematical representations, which attempt to reproduce the behaviour of the Earth’s climate system (Trenberth 1992). As outlined in Sect. 2.1 the climate system is composed of a number of subcompartments (e.g. the atmosphere, hydrosphere, cryosphere, and biosphere), each component is a non-linear system in itself and is associated with a characteristic time scale.

The representations of climate models attempt to account for the most important external and internal forcings, they also try to include the most important processes which are involved in feedbacks within the system. The models are based on physical principles such as the conservation of energy, momentum, and mass. Although the fundamental principles appear to be robust, computational limits preclude their numerical solution on all scales which include the many processes that are important in the climate system. For these processes, modellers have to rely on parameterisations which try to capture the fundamental phenomenology of a small scale process. For more details on the state-of-the-art of climate modelling see, e.g. Washington and Parkinson (2005), Kiehl and Ramanathan (2006), or Donner and Large (2008).

Current climate models simulate the atmosphere, with a prescribed atmospheric composition and incoming solar radiation, and include explicit modelling of the ocean's general circulation and sea-ice dynamics, and relevant land processes. By way of interfaces the subcomponents can exchange energy, momentum, and mass. A typical horizontal grid resolution of today's global atmospheric models is about 180 km; regional climate models on the other hand, capture only part of the globe, and typically work with a 25–50 km horizontal grid resolution. In the vertical dimension, the resolution decreases with the distance from the Earth's surface and ranges from a few decametres to a few kilometres. Among the most important feedbacks climate models have to cope with is the water vapour and the cloud feedbacks as well as the snow, sea and land-ice albedo feedbacks, which also need to be mentioned. Cloud feedbacks can be substantial for the overall performance of a climate model, but the sign and the magnitude of the global mean cloud feedback depends on so many factors that it remains very uncertain (Stephens 2005; Bony et al. 2006).

Climate models are far from perfect, but they are unmatched in their ability to quantify otherwise qualitative hypotheses. More than a dozen facilities worldwide develop and maintain state-of-the-art climate models and comprehensive model comparisons have been conducted using the present-day climate as a primary test bed. These models have successfully captured fundamental aspects of air, ocean, and sea-ice circulations as well as their variability. In recent climate studies (i.e. IPCC 2007a), results of ensembles of a number of models have been reported (such as outlined in Sect. 5.3.). Interestingly however, comparison studies have revealed that the average across a number of models outperformed any given single model. Climate models are evolving more and more into Earth system models, which include chemical and biological processes (e.g. vegetation dynamics) as well as simple socio-economic sub-models.

## 5.2 *Emission Scenarios*

A scenario is a description of potential future conditions produced to inform and assist in what would otherwise be decision-making with an even greater uncertainty.

However, more than just a reliable model of the physical climate system is needed for climate projections of the future. Here, there is also the need to supply the models with estimates of the development of the forcing parameters. Of central interest are future anthropogenic modifications of atmospheric greenhouse gasses and aerosol concentrations as well as characteristics of the Earth's surface. The IPCC has produced three sets of scenarios of twenty-first century greenhouse-gas emissions, the most ambitious and important of which were produced for the *Special Report on Emissions Scenarios* (SRES) (IPCC 2000). SRES produced four qualitative storylines on which six “marker” scenarios were based – one model quantification of each storyline plus two technological variants of one storyline which stressed fossil-intensive and low-carbon energy supply technologies – and each produced by a different energy-economic model. Scenarios are descriptions of (different) possible futures, they are a series of alternative visions of the future (storylines), which are possible, plausible, internally consistent but *not* necessarily probable (von Storch 2008). The information which is really required is based on scenarios of emissions of radiatively active substances, and these scenarios themselves depend on a variety of future developments in different sectors and in particular on population growth, changes in technology and the energy system and its efficiency. Many of these factors are not easy to predict and so a variety of assumptions have entered into these scenarios. It is not surprising, therefore, that critical discussions arose as a result (e.g. Tol 2008; Pielke et al. 2008). The SRES scenarios do not include additional climate initiatives, which means that no scenarios are included which explicitly assume the implementation of the United Nations Framework Convention on Climate Change or the emissions targets of the Kyoto Protocol. In other words, these scenarios do not anticipate any specific mitigation policies for avoiding climate change. The IPCC scenarios are grouped in the following way:

(A1) represents a world of rapid economic growth and a rapid introduction of new and more efficient technology. The A1 storyline and scenario family describes a future world of very rapid economic growth, a global population which peaks in mid-century and declines thereafter and the rapid introduction of new and more efficient technologies. Major underlying themes are convergence among regions, capacity building and increased cultural and social interactions, with a substantial reduction in regional differences in per capita income.

(A2) represents a very heterogeneous world with an emphasis on family values and local traditions (high-CO<sub>2</sub>). The A2 storyline and scenario family describes a very heterogeneous world. The underlying theme is self-reliance and preservation of local identities. Fertility patterns across regions converge very slowly, which results in a continuously increasing population. Economic development is, primarily, regionally oriented and per capita economic growth and technological change are more fragmented and slower than in other storylines.

(B1) represents a world of “dematerialization” and the introduction of clean technologies (low-CO<sub>2</sub>). The B1 storyline and scenario family describes a convergent world with the same global population, which peaks in mid-century and declines thereafter, as is the case in the A1 storyline, but with rapid change in

economic structures toward a service and information economy, with reductions in material intensity and the introduction of clean and resource-efficient technologies. The emphasis is on global solutions to economic, social and environmental sustainability, including improved equity, but without additional climate initiatives.

(B2) represents a world with an emphasis on local solutions to economic and environmental sustainability. It is a world with a continuously increasing global population, at a rate lower than A2, intermediate levels of economic development, and less rapid and more diverse technological change compared to the B1 and A1 storylines. While the scenario is also oriented towards environmental protection and social equity, it focuses on local and regional levels.

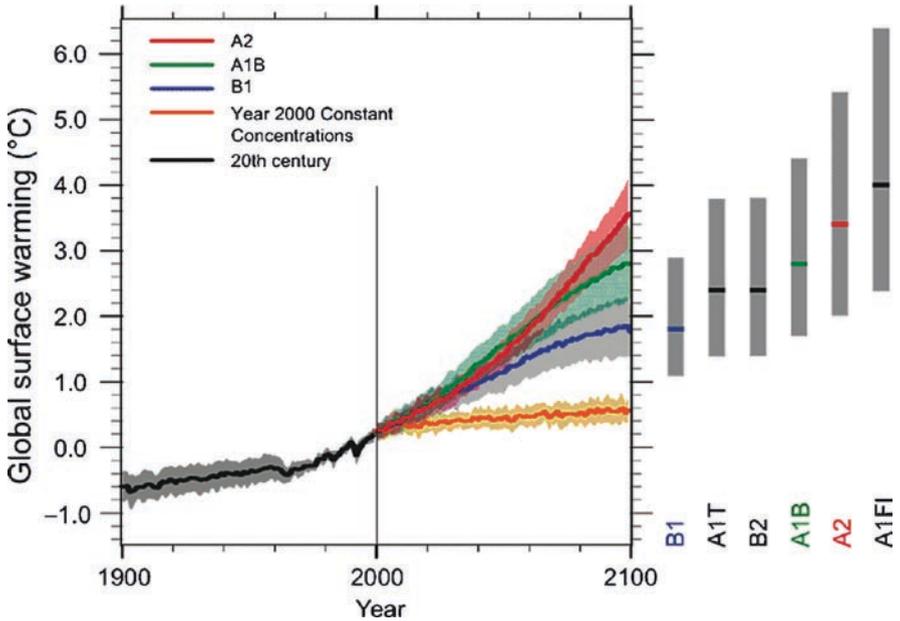
An illustrative scenario was chosen for each of the six scenario groups A1B, A1FI, A1T, A2, B1 and B2, and all should be considered equally sound. The A1 scenario is broken down into some variants: in recent years, the A1B (balanced – not relying too heavily on one particular energy source and similar improvement rates for all energy supply and end-use technologies) was widely used together with the B2 scenario. A1F represents a world, which still relies heavily on the use of fossil fuels (worst case) and A1T is a scenario, which incorporates the use of advanced technologies. A selection of results presented in the next section will refer to these scenarios.

### 5.3 *Climate of the Twenty-First Century*

For about two decades now, coupled ocean–atmosphere climate models have been in use for making sophisticated climate change projections for the current century. Most of the published model studies are reviewed by the successive assessment reports of the IPCC starting in 1990. Over the years, the underlying simulations have become more advanced, either through the inclusion of additional dynamic sub-models, improved parameterisations or increased spatial resolution. The results of the newest simulations are the basis of the climate projections and impacts discussed in the fourth IPCC assessment report (IPCC 2007a,b). The results presented in this section will, to a large extent, build on these studies.

#### 5.3.1 **Temperature**

One of the most communicated graphs of the IPCC AR4 is reproduced in Fig. 9. The graph shows the time evolution of the multi-model global-mean surface temperature in transient emission scenario driven experiments according to the most comprehensive model study. As can be seen in Fig. 9, the projected temperature changes depend decisively on the underlying scenario. Based on all six scenarios and taking the model ranges, expressed by the vertical bars on the right hand side of Fig. 9, within single scenarios into account, global warming between 1.8°C



**Fig. 9** Multi-model means of surface warming (relative to 1980–1999) for several scenarios A2, A1B and B1, shown as continuations of the 20th-century simulation. Lines show the multi-model means, shading denotes the  $\pm 1$  standard deviation range of individual model annual means. Note that not all scenarios are run by the same number of models, thus different standard deviations are not suited for interpretation across scenarios (IPCC 2007a)

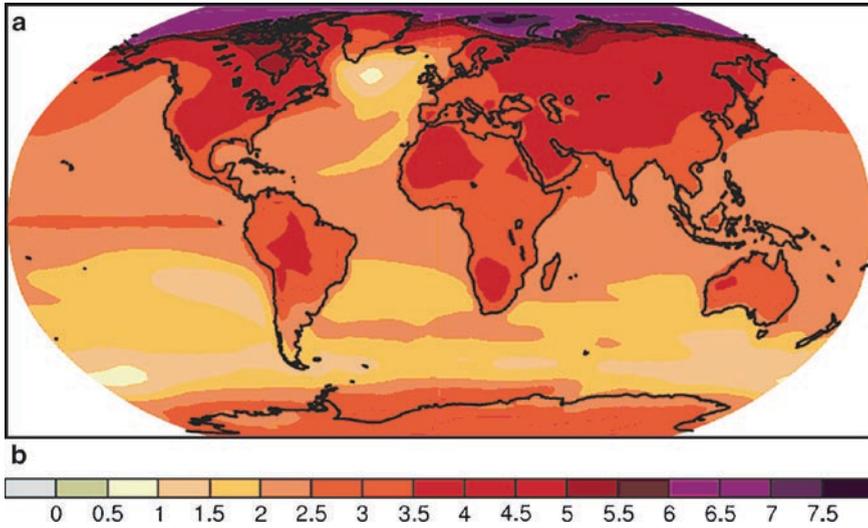
(1.1–2.9°C) for B1 and 4.0°C (2.4–6.4°C) for A1FI has to be anticipated by the end of the twenty-first century (2090–2099 relative to 1980–1999). In the underlying climate simulations the observed temperature changes of the last century are reproduced (left part of Fig. 9) and amplified in the course of the twenty-first century. Uncertainty in the climate projections is due not only to the unknown path society will take with respect to socio-economic aspects and technological development, as expressed by the scenarios, but also to considerable differences between the participating models. These inter-model differences are, to a large extent, due to the representation of feedback processes in the atmospheric general circulation models, and here especially the cloud feedback (Bony et al. 2006). However, all the models show a considerable warming by the end of the century, and even climate simulations keeping the same greenhouse gas concentrations as the year 2000 show an increase in global mean temperature of another half of a degree, reflecting the thermal inertia in the climate system. Table 1 provides the numerical values for global warming at the end of the century. The best estimate as well as the likely range from the multi-model evaluation for the different scenarios are given.

The geographical distribution of climate change is of paramount interest for many climate impact studies. Considerable spatial variation exists in modelled

**Table 1** Projected global average surface warming at the end of the twenty-first century ((in °C at 2090–2099 relative to 1980–1999)

Scenario	Best estimate	Likely range
B1	1.8	1.1–2.9
A1T	2.4	1.4–3.8
B2	2.4	1.4–3.8
A1B	2.8	1.7–4.4
A2	3.4	2.0–5.4
A1FI	4.0	2.4–6.4

Data from IPCC (2007a)



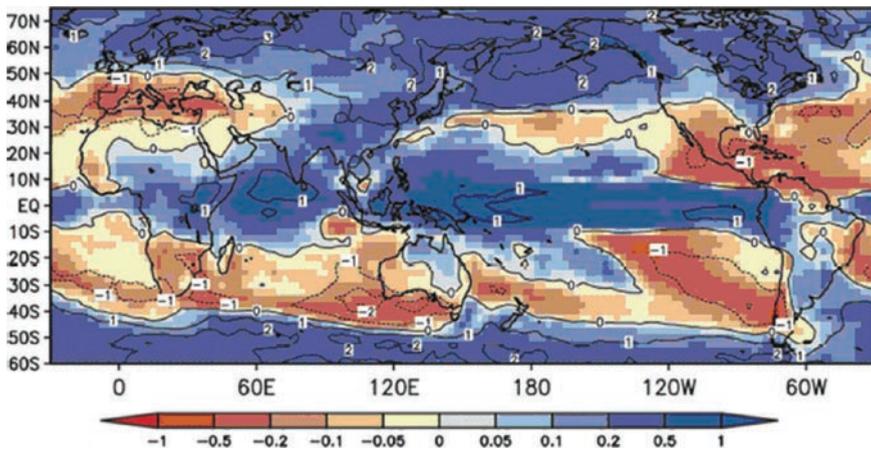
**Fig. 10** Multi-model mean of annual mean surface warming (surface air temperature change relative to 1980–1999 in °C) for the scenario A1B (medium emission scenario) and the time period 2080–2099. Figure extracted from IPCC (2007a)

changes in climate. There is a certain pattern of climate change over the globe, which is consistently reproduced by all contributing models. As an example, the multi-model surface temperature distribution at the end of the century resulting from scenario A1B runs is shown in Fig. 10; all other scenario simulations yield the same principle pattern. In general, however, continents will warm faster than the oceans. The strongest warming is evident over the Northern land masses and the polar regions due to the positive ice-albedo effect. In these regions, the warming trend is about twice that of the global mean, and is similar therefore to that observed for the previous century. The lowest temperature change might be expected over the Southern oceans in the latitude band between 40° and 50°S and over North Atlantic ocean. Oceans respond with a delay, since the vertical mixing distributes the additional heat over a much larger volume compared to land masses. Vertical mixing is especially efficient in the North Atlantic and the Southern Ocean. It has to be mentioned, that there are remarkable differences between the single models when

regional temperature distributions produced by the global models are more closely inspected (Fig. 10).

### 5.3.2 Precipitation

The direct implications of global warming are higher evaporation rates and therefore, higher precipitation rates. As a global and multi-model average, an increase in precipitation of about 4–5% can be expected towards the end of the twenty-first century (Meehl et al. 2005), with a net change over land accounting for 24% of the global mean increase in precipitation. It should also be pointed out that for precipitation over land especially, large inter-model deviations exist, and these occur not only in the regional mean but also in the global mean. Figure 11 shows the distribution of changes in the annual mean precipitation as a scenario A1B multi-model mean expressed in mm per day for the last two decades of the present twenty-first century. It can be seen that a large general increase in precipitation is not to be expected, but that there will be regions on the globe where more water will be available and others where drying might occur. More rain is simulated for the equatorial belt and higher geographical latitudes. Less precipitation (up to 20%) is projected especially for the subtropics (oceans, Mediterranean sea, South Africa, Australia). This projected decrease is considered especially robust, and *very likely* to occur (IPCC 2007a). From the results of inter-annual evaluations of the model a shift in the timing and strengths of precipitation events has been diagnosed, and this could be of huge importance for the future management of drinking water supply and the fate of ecosystems where water is critical.



**Fig. 11** Mean changes in precipitation (in mm day<sup>-1</sup>). Changes are annual means for the A1B scenario for the period 2080–2099 relative to 1980–1999. Figure from Daisuke Nohara

### 5.3.3 Sea Level Rise

It can be considered as a robust result of the IPCC Model projection that the sea level will continue to rise in the twenty-first century because of thermal expansion and loss of land ice. Sea level rise was not geographically uniform in the past and will not be in the future. Projected warming due to emissions of greenhouse gases during the twenty-first century will continue to contribute to sea level rise for many centuries to come. Sea level rise due to thermal expansion and loss of mass from ice sheets will continue for centuries or millennia even if the radiative forcing were to be stabilised. Table 2 provides the numerical values for the sea level rise at the end of the century, and the likely range from the multi-model evaluation for the different scenarios is also provided. The range excludes future rapid dynamical changes in ice flow, which is not parameterized in the current ice sub-models.

In all scenarios, the average rate of sea level rise during the twenty-first century is *very likely* to exceed the 1961–2003 average rate ( $1.8 \pm 0.5 \text{ mm year}^{-1}$ ). During 2090–2099 under A1B, the central estimate of the rate of rise is  $3.8 \text{ mm year}^{-1}$ . For an average model, the scenario spread in sea level rise is only 0.02 m by the middle of the century, and by the end of the century it is 0.15 m. Thermal expansion is the largest component, contributing 70–75% of the central estimate in these projections for all scenarios. Glaciers, ice caps and the Greenland ice sheet are also projected to contribute positively to sea level. The Antarctic Ice Sheet will receive increased snowfall without experiencing substantial surface melting, thus gaining mass and contributing negatively to sea level. Further accelerations in ice flow of the kind recently observed in some Greenland outlet glaciers and West Antarctic ice streams could substantially increase the contribution from the ice sheets (IPCC 2007a). 2005 was the deadline for scientific input into the IPCC AR4 report; however, there has been some further work carried out on sea level rise which was motivated by the observed acceleration of the Greenland ice melting. The models used for the AR4 sea level assessment have certain shortcomings concerning the small scale processes in melting physics. All newer estimates of the possible sea level rise towards the end of the twenty-first century come up with higher estimates compared to those reported in IPCC (2007a). For example, Rahmstorf (2007) uses a semi-empirical relation which connects global sea-level rise to global mean surface temperature in the twentieth century. The use of such a semi-empirical relationship is, however, not without its critics. When the established correlations are applied to

Scenario	Likely range
B1	0.18–0.38
A1T	0.20–0.45
B2	0.20–0.43
A1B	0.21–0.48
A2	0.23–0.51
A1FI	0.26–0.59

**Table 2** Projected global sea level rise at the end of the twenty-first century (in m between 2090–2099 relative to 1980–1999; model-based range excluding future rapid dynamical changes in ice flow)

Data from IPCC (2007a)

the future warming scenarios of the IPCC, the relationship results in a projected sea-level rise in 2100 of 0.5–1.4 m above the 1990 level. A comparison of the past sea level rise from studies of the last interglacial period with presently projected conditions, led Rohling et al. (2008) to the statement “We find average rates of sea-level rise of 1.6 m per century. As global mean temperatures during MIS-5e were comparable to projections for future climate change under the influence of anthropogenic greenhouse-gas emissions, these observed rates of sea-level change inform the ongoing debate about high versus low rates of sea-level rise in the coming century”. An extreme possible sea level rise of up to 5 m is brought into discussion by Hansen (2007c) who also refers to palaeoclimate records containing numerous examples of ice sheets disintegrations yielding sea level rises of several metres per century in situations when forcings were smaller than that of the business-as-usual scenario (close to SRES A2).

Overall, it should be stated that future sea level rise is still very uncertain, but the numbers given in the IPCC AR4 seem to mark the absolute lower boundary of possibilities, and a possible rise in sea level of a meter or slightly more by the end of the century seems to be more likely.

### 5.3.4 Extreme Events

Recent years have seen a number of weather events causing large losses of life (Easterling et al. 2000b). Climate model improvements have resulted in an enhanced ability to simulate many aspects of climate variability and extremes. However, they are still characterized by systematic errors and limitations in accurately simulating regional climate conditions. Nevertheless, an analysis of the frequency of occurrence of selected parameters have shown that besides a shift in the mean, a broadening of the probability density distribution is also to be expected, thereby implying a higher variability and more extreme values. Heat waves will become more frequent and longer lasting in a future warmer climate (IPCC 2007a).

Extremes of daily precipitation are *likely* to increase in many regions. The increase is considered as *very likely* in Northern Europe, South Asia, East Asia, Australia and New Zealand. Multi-model climate projections for the twenty-first century show both increases in precipitation intensity and the number of consecutive dry days in many regions (Tebaldi et al. 2006). Precipitation intensity increases almost everywhere, but particularly at mid- and high latitudes, where mean precipitation also increases. There are many regions in the sub-tropics and lower mid-latitudes with an increased number of consecutive dry days, the sign of this parameter is reversed at higher mid-latitudes and high latitudes, where mean precipitations are expected to increase.

Although somewhat counter-intuitive, a projected increase in the risk of intense precipitation and flooding is also associated with the risk of drying. Intense and heavy episodic rainfall events with high runoff are interspersed with longer relatively dry periods with increased evapotranspiration especially in the sub-tropics (Bates et al. 2008).

### 5.3.5 Regional Scale Information: Dynamical Downscaling

Downscaling is a method which derives local-to-regional-scale information from larger-scale models. The dynamical downscaling method uses the output from global climate models, which typically run on a coarse resolution, as a driver for regional climate models, which again can be setup in a nested manner down to the desired spatial resolution. The dynamical model on the regional scale allows for the added value of the development of meteorological phenomena, which evolve because of regional flow and surface flux features. Of course, the quality of the downscaled product depends to a very large extent on the quality of the driving model.

It is beyond the scope of this overview to discuss all the results from the published regional climate projection studies; however, some major references will be provided here. For example, Räisänen et al. (2004) and Giorgi et al. (2004), and Bhend and von Storch (2008) each highlight different aspects of climate change projections for entire Europe. In the following, a few selected regional results are summarized:

#### Alps

Raible et al. (2006) evaluated reconstructed data series from the past and performed climate model projections for the Atlantic-European and Alpine region. Time-slice simulations using a regional model were carried out. In the A2 scenario simulation (2071–2100) the winter and summer temperatures show an average increase of about 3 and 4°C, respectively. Comparing the control time-slice 1961–1990 with the A2 scenario, the interannual variability is unchanged in winter, whereas a strong increase on these time scales in summer is observed (Schär et al. 2004). Precipitation behaves differently; in winter, a small average increase is simulated in the A2 scenario. The simulated trend in winter precipitation for the control period 1961–1990 is consistent with the observed trend and the reconstruction; thus this behaviour may be explained by internal variability. In summer, the mean precipitation shows a small decrease compared with the mean of the control simulation. Comparing the A2 with the control simulation, the inter-annual variability of precipitation remains unchanged in winter, but increases in summer.

#### Baltic Region

The climate projections reported in BACC (2008) focussed on an *Assessment of Climate Change in the Baltic Sea Basin*. The main results are on the time horizon of 2100, and show that without mitigation measures the surface temperatures are expected to rise by about 4–6°C in the Northern Baltic region and by about 3–5°C in the Southern part of the Baltic including parts of Poland and Northern Germany. This temperature increase will lead to a reduction in the winter ice cover of the

Baltic Sea by 50–80%. Winter precipitation is projected to rise by 25–70% and in summer precipitation is expected to decrease up to 45% by the end of the century.

### Extremes Over Europe

Beniston et al. (2007) evaluated regional climate model projections for future extreme events in the European climate. They explored how heat waves, heavy precipitation, drought, wind storms, and storm surges change between the present (1961–1990) and future (2071–2100).

The main results of the study can be summarized as follows: Heat waves – regional surface warming causes the frequency, intensity and duration of heat waves to increase over Europe; Precipitation – heavy winter precipitation increases in central and Northern Europe and decreases in the South; heavy summer precipitation increases in North-Eastern Europe and decreases in the South. Mediterranean droughts start earlier in the year and last longer; Winter storms – extreme wind speeds increase between 45° N and 55° N, except over and South of the Alps, and become more North-westerly than they are at present.

Schär et al. (2004) simulated possible future European climate using a regional climate model and a scenario with increased atmospheric greenhouse-gas concentrations. The assessment of European heat waves in relation to the extreme event in 2003 (strongest heat wave for more than 500 years) was at the centre of the study. The authors found that under a certain emission scenario (SRES A2) temperature variability over Europe increases by up to 100%, with maximum changes in central and Eastern Europe. The European summer climate may also experience a pronounced increase in year-to-year variability in response to greenhouse-gas forcing. The regional climate model simulations suggest that towards the end of the century – under the given scenario assumptions – about every second summer could be as warm or warmer (and as dry or dryer) than that of 2003. Thus, the conditions experienced during the serious heat wave of 2003 will be typical summer conditions by the end of the century.

Also, Diffenbaugh et al. (2007) found that elevated greenhouse gas concentrations dramatically increase heat stress risk in the Mediterranean region, with the occurrence of hot extremes increasing by 200–500% throughout the region.

The work of Räisänen et al. (2004), for example, and model evaluation work by the present author underpin the idea that the quality of the driving models predominantly determine the output of the regional climate models. This statement holds especially for water cycle quantities (Graham et al. 2007). In this case, the differences between the results obtained using two driving models coupled to one regional climate model with each simulating the same emission scenario is larger than those observed between one large scale model and one subsequent regional model simulating two extremely opposite scenarios. With the present-day suite of models it has to be stated that regional climate change results derived using only one driving model feeding one regional model need to be interpreted with caution.

### 5.3.6 Abrupt Changes

The Earth's climate system is highly non-linear: inputs and outputs are not proportional, change is often episodic and abrupt rather than slow and gradual, and multiple equilibria are the norm (Rial et al. 2004). Most of the studies and debates on potential climate change, along with its ecological and economic impacts, have focused on the ongoing build-up of industrial greenhouse gases in the atmosphere and a following *gradual* increase in global temperatures. This line of thinking, however, fails to consider another potentially disruptive climate scenario. It ignores recent and rapidly advancing evidence that the Earth's climate has repeatedly shifted *abruptly* and *dramatically* in the past, and is capable of doing so in the future. Fossil evidence clearly demonstrates that the Earth's climate can shift gears *within a decade*, establishing new and different patterns, which can persist from decades to centuries (NRC 2002).

The evidence also shows that the Earth's climate system has sensitive thresholds. Pushed past these thresholds and the system can jump quickly from one stable operating mode to a completely different one. These thresholds have been communicated in recent literature under the concept of "tipping points" (Lenton et al. 2008).

Many triggers for abrupt changes have been identified in the climate system and some of them are discussed in NRC (2002), Alley et al. (2003), and IPCC (2007a). One of the more prominent effects of abrupt changes is related to the deep water formation in the Northern Atlantic. The past 30 years have witnessed an intrusion of low-salinity deep waters which spread over the entire sub-polar North Atlantic Ocean and the seas between Greenland and Europe, and in just those regions which are critical for abrupt shifts in the thermohaline circulation, which has been implicated in many abrupt climate-change events in the past (Alley et al. 2003).

A salient feature of terrestrial climate change is its *asymmetry*. Warming is rapid, usually followed by a slower descent into colder climate. Given the symmetry of orbital forcings, the cause of rapid warming at glacial 'terminations' must lie in a climate feedback. Clearly, the asymmetric feedback is the albedo flip of ice and snow which occurs when they become warm enough to begin melting (Hansen et al. 2007a). A climate forcing which 'flips' the albedo of a sufficient portion of an ice sheet can initiate a cataclysm. This positive feedback can lead—and has led—to a relatively fast increase in surface temperatures.

## 5.4 Climate on the Millennium Time Scale

The present chapter will close with a short outlook on the projected climate change for the actual millennium. Most of the published climate projections address the twenty-first century and only a few studies exist, which look further into the future. Because of the considerable inertia of the climate system – caused by the long residence times of many greenhouse gases, the large heat capacity of the oceans and the long-term memory of other components of the system, such as ice sheets – in

the absence of appropriate mitigation measures, human modifications of the climate system are expected to persist for many centuries to come. For example, it is likely that a substantial fraction of the projected CO<sub>2</sub> emissions will stay in the atmosphere for millennia and therefore, the related climate changes are to be expected in the longterm (Archer and Brovkin, 2008).

Hasselmann et al. (2003) report on simulations up to the year 3,000 using a non-linear impulse response climate model coupled to an elementary economical model. Their results depend strongly on the assumed emission scenarios, for estimated peak CO<sub>2</sub> concentrations between about 1,200 and 4,000 ppm, and considering the use of all conventional and exotic resources, global temperature increases of 4–9°C are predicted for the second half of this millennium. The subsequent sea level rise will range from 3 to 9 m. The conclusion of Hasselmann et al. (2003) is that the global warming anticipated in the following centuries is a potentially far more serious problem compared to projected climate change in this century, i.e. if greenhouse gas emissions continue to increase unabated.

Based on simulations with eight Earth system models of intermediate complexity (climate-carbon cycle models) and different emission scenarios with assumed stabilized greenhouse gas concentrations after the year 2,100, Plattner et al. (2008) assess global temperature changes and sea level rise up to the year 3,000. The authors have identified a substantial climate change commitment for global temperature increase and sea level rise even after the stabilization of atmospheric greenhouse gas concentrations. For an initially high-CO<sub>2</sub> A2 plus stabilization scenario, the additional warming by the year 3,000 is calculated to range from 1.3 to 2.2 K and for a low-CO<sub>2</sub> B1 plus stabilization scenario from 0.6 to 1.6 K. The sea level continues to rise due to thermal expansion and the respective values for the A2 and B1 scenarios are 0.5–5.5 m and 0.3–1.1 m. The climate-carbon cycle models revealed that by the year 3,000 most of the anthropogenic carbon emissions will be eventually taken up by the ocean, but that a substantial fraction (15–28%) will still remain in the atmosphere. Even if the carbon emissions are hypothetically reduced to zero after the year 2,100, sea level will rise further due to ongoing thermal expansion. In this instance, a rise of 0.1–0.7 m by the year 3,000 has been calculated. Global surface temperatures remain high in the zero-post- 2,100 scenario, and compared to the year 2,100 a reduction of only a few tenths Kelvin can be expected by the year 3,000.

In summary, it can be said that emissions from the twenty-first century will *noticeably* affect the climate over the entire millennium.

## 6 Concluding Remarks

Over the 4.6 billion years of its evolution, the Earth has seen a large variety of climate states, which in their extremes have ranged from either the *snowball Earth* or *hot house*. Extended periods of an intermediate climate with only moderate changes have also been deduced from proxy data. However, climate variability is

the *rule* and not the exception and the evolution of life on Earth was closely linked to climate and its change (Kasting and Catling 2003).

For about 250 years, mankind has interfered stronger with the climate system in the sense that, in addition to land use changes, radiative active gases and particles have been released in substantial amounts into the atmosphere. Over this period especially, the atmospheric concentrations of the greenhouse gases CO<sub>2</sub> and methane have risen significantly and account for an additional radiative forcing of more than 2 W m<sup>-2</sup> (IPCC 2007a). A global mean temperature increase can be deduced from instrumental observations for the same time span. The recent warming recorded in the instrumental observations over the last decade exceeds the temperatures (including the uncertainty ranges) of at least the past 1,600 years, as reconstructed for the Northern hemisphere from an expanded set of proxy data (Mann et al. 2008). Climate model projections driven by greenhouse gas and particle emissions according to a set of socio-economic scenarios emphasise a further increase in global temperature for the present century and beyond (IPCC 2007a). These projections encouraged some scientists to speak of a dangerous interference with the climate system, which could place the Earth perilously close to dramatic climate change that could run out of our control, resulting in great dangers for humans, other creatures and ecosystems (Hansen et al. 2007a,b). Based on the likelihood of strong El Niños in the future, Hansen et al. 2006 argue that a global warming of more than about 1°C, relative to the year 2000, would constitute a “dangerous” climate change for some regions, as judged from the likely effects on sea level and extermination of species. Although the term “*dangerous interference*” holds some subjective notion, there does appear to be a widely acknowledged awareness that, besides emission mitigation actions, adaptation measures have to be implemented.

Even if there have been hotter times in the deep history of our planet, there are two aspects which enhance the relevance of the current change in temperature and other climate parameters. Firstly, there are many *more people* than ever which live in regions of the world which are vulnerable to climate impacts. Secondly, the *pace* of the temperature increase in relation to the amplitude is almost unprecedented in the analysed climate records and will lead to a geographical shift in climate conditions, which may make it difficult for some ecosystems to adapt to or for some species to migrate in time (IPCC 2007b).

There are a few climate elements which may be sensitive to sudden, abrupt changes when the boundary of a set of conditioning parameters is overstepped or when certain thresholds are passed. Large, abrupt, and widespread climate changes with major impacts have occurred repeatedly in the past (Alley et al. 2003). An often cited example of an abrupt change is that of a possible collapse of the thermohaline circulation originating in the North Atlantic.

The discussion on abrupt changes has, generally, only considered the implications from an economic and ecological points of view; however, Hulme (2003) also discusses the implications from a more *social* and *behavioural* science perspective. Although abrupt changes might be of enhanced relevance for the global society, a detailed knowledge of the geophysical processes involved is *not* complete and a scientific consensus regarding the overall relevance and expected timing has *not* yet

been fully reached. Therefore, extending our research into the area of abrupt climate change is urgently needed. Lenton et al. (2008) define tipping points in the climate system and report transition times of between 10 and 100 years. Among the discussed tipping elements are those of the thermohaline circulation, the Arctic sea-ice, the Greenland and West Antarctic ice sheets, the Indian summer monsoon, the Sahara/Sahel, West African monsoon and the Amazon rainforest (not the complete list). Lenton et al. (2008) also note that society may be lulled into a false sense of security by smooth projections of global change. According to their study, a synthesis of our present knowledge suggests that a variety of tipping elements could reach their critical point within this century. In their more general analysis, Steffen et al (2007) conclude with the statement: “Whatever unfolds, the next few decades will surely be a tipping point in the evolution of the Anthropocene”.

**Acknowledgement** The author thanks Dr. Jan Habel and Professor Thorsten Assmann for having given the opportunity to contribute to the symposium on relict species in Luxembourg. Prof. Raymond Pierrehumbert, University of Chicago, USA, Prof. Leo Donner, Princeton University, USA, and Dr. Victor Brovkin, MPI for Meteorology, Hamburg, Germany, provided yet unpublished material, thank you all for the kindness. Valuable comments on the manuscript provided by Dr. Desmond Murphy are gratefully acknowledged. A part of the work is based on lectures on environmental meteorology and climate physics by the author at the University of Lüneburg, Germany.

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# Extra-Mediterranean Refugia, Post-Glacial Vegetation History and Area Dynamics in Eastern Central Europe

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**Abstract** Evidences from fossil records and genetic research suggest that the arboreal refugia were not restricted to Southern Europe and in particular to the Mediterranean peninsulas during the full-glacials. Fossil pollen data and macrofossil remains indicate that several tree species have survived also at the Southern edge of the cold-dry steppe-tundra area in Central and Eastern Europe. Recent results of surveys on the Late Pleistocene Mammalian fauna clearly contradict to the “*tree-less tundra*” models for Europe North of the transverse mountain ranges of the Pyrenees, Alps and Carpathians. It was pointed out that the carrying capacity to feed the herds of large herbivores demands a rather productive environment. The presence of Northern temperate refugia is also supported by the “non-analogue” assemblages of small mammals discovered from the Late Pleistocene of unglaciated areas of Eastern Central Europe. The assembly of species today typifying the tundra, steppe and semi-desert habitats seems to include also species from deciduous woodland. Extra-Mediterranean core areas were identified also in widely dispersed cold-tolerant frogs and reptiles. Some of their core areas had been at least near the Carpathians and/or marginal areas of the Carpathian Basin. The close faunal connections of the Carpathians suggest the existence of highly dynamic contacts and exchanges with mountains of the Balkan Peninsula during the climatic fluctuations of the Upper Pleistocene. The Eastern and Southern Carpathians, together with the mountains of Western Transylvania, can be considered as core areas of survival and autochthonous evolution in some invertebrate groups with limited mobility. The post-glacial re-population of the Carpathian Basin from different directions has been supported by Illyrian versus Dacian vicarious pairs of sister species/subspecies. In mobile insect groups, peripherically isolated sibling species/subspecies have only been evolved, which display manifold biogeographic connections, e.g. to the Balkan Peninsula, Asia Minor or Southern Russia. The organisation of community-complexes of the Pannonian forest-steppe

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connected by habitat ecotones resulted in the overlap of several different faunal types, e.g. Mediterranean, Balkanic, Siberian, Ponto-Caspian, Ponto-Pannonian, Turano-Eremitic and Xeromontane elements.

## 1 Introduction: Refugia and Core Areas of Dispersal

### 1.1 *The Basic Components of the European Fauna*

Traditional biogeography has been loaded with a seemingly endless discussion on the principles and methods of the biogeographical characterisation of species. In the geobotanical literature, the purely chronological (*geoelements*) and the historical approaches (*geno-*, *migro-* and *chrono-elements*) have regularly been clearly disentangled (e.g. Walter and Straka 1970). In the zoogeographical literature, the term “faunal element” or “faunal type” has repeatedly been used for both approaches, often with a mixture of points of view. Faunal types have often been differentiated on the basis of the shape and the extension of the ranges (e.g. the “Central European”, “Palearctic” or “Holarctic”, etc. faunal elements in many faunistic publications). Quantitative methods of biogeography based on large databases have only rarely been used. Dennis et al. (1991) differentiated some types of endemic versus “extent” (i.e. widespread) species of butterflies, and have outlined some “*faunal structures*”, based on multivariate methods. As opposed to these methods, the European fauna has traditionally been subdivided into a “*holothermic*” refugial and a “*holopsychric*” invasion types (Rebel 1931). In addition, these faunal types have been characterised by some “*core areas*” (in German: *Arealkerne*, *chorologische Zentren*: Reinig 1950; de Lattin 1957, 1967) and interpreted as areas of survival (refugia) and, consequently, as “*centres of dispersal*” (“*Ausbreitungszentren*” in the German tradition).

The “*holothermic*” type was differentiated according to the secondary subdivision of the large Mediterranean refugial area (de Lattin 1949, 1957, 1967; Reinig 1950). Recently, this view was strongly confirmed and modulated by the growing molecular results and re-formulated as repetitive patterns of the generalised “paradigms” of core areas and tracks of post-glacial re-population. A general conclusion was that temperate species mainly derive from Mediterranean refugial populations that underwent range expansion in the late glacial and early post-glacial periods (Hewitt 1996, 1999, 2000, 2001, 2004; Taberlet et al. 1998; Schmitt and Hewitt 2004; Schmitt 2007). The other main group, the “*holopsychric*” type has been considered for a long time as a result of the “Siberian” invasion, suggested by some “classical”, monoglacialisitic biogeographical works (Hofmann 1873; Scharff 1899) and despite the evidences which have revealed the taxonomical differentiation of North-Eastern “boreal” and Southern European montane populations, especially in some butterfly species (Varga 1975, 1977; Nève 1996). Several authors (e.g. Schmitt and Seitz 2001; Steward and Lister 2001; Surget-Groba et al. 2001; Babik et al. 2004;

Ursenbacher et al. 2006; Saarma et al. 2007), however, have suggested an additional mode of colonisation of central and Northern Europe by *non-Mediterranean* populations, coming from one or more “continental” refugia: central Europe, Southern Ural, Caucasus and Western Asia. Other species exhibit mixed patterns, where different parts of Europe have been colonised both from Mediterranean and/or non-Mediterranean refugia (e.g. Fumagalli et al. 1996; Deffontaine et al. 2005; Kotlík et al. 2006).

## 1.2 *The Geographical Projection of the Faunal Type Frequency Data*

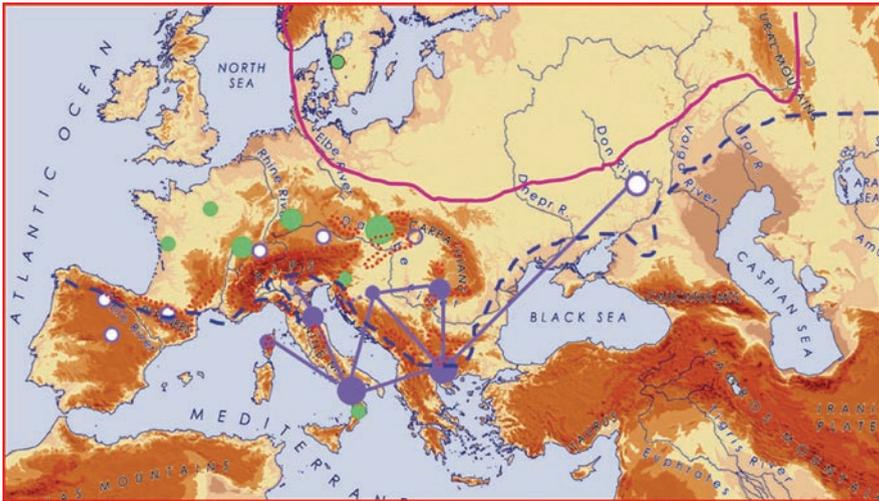
The faunal composition of formerly glaciated and therefore, nearly exclusively post-glacially re-populated Northern Europe is practically identical with North-Eastern Europe and Western Siberia which were considered as a main argument for being re-populated mostly from the East (Rebel 1931; de Lattin 1957, 1967). This major part of Europe clearly shows a high percentage of the “Siberian” faunal type in all mobile groups of animals such as birds (Stegmann 1932, 1938; Voous 1960, 1963) and butterflies (Reinig 1950; de Lattin 1964, 1967, see: Fig. 59, in Kostrowicki 1969; Fig. 1). This faunal type is also strongly represented in Central Europe, North of the Alps, being the prevailing faunal type mostly in the mountainous regions (e.g. Harz Mts., Bavarian and Bohemian forest, Sudetic Mts., etc.). In contrast, South of the large transversal chains of the Pyrenées, the Alps and the Northern Carpathians, the pre-dominance of the Mediterranean faunal type *sensu lato* has been demonstrated, with a decreasing gradient into Northern direction being extremely steep at the Pyrenees and partly North of the Alps and the Carpathians, but much more gently sloping from the Balkan peninsula to the Carpathian Basin. As a consequence of the overlapping of the different biogeographical influences, there is a transitional belt in Southern Central Europe including a large part of the Carpathian Basin where the proportions of the different faunal types are rather balanced (de Lattin 1967; Varga and Gyulai 1978; Varga 1995, 2003b, 2006).

Several consequences follow from this general biogeographical setting of Europe.

- In Europe, the highest number of endemic species is confined to some, mostly mountainous parts of the Mediterranean peninsulas (e.g. Williams et al. 1999; Finnie et al. 2007). These areas, more or less, regularly overlap in the different taxonomical groups; thus they can be considered as hotspots for endemism, and at the same time, they were the main areas of survival during the Quaternary glaciations. These areas often show also a high level of “multi-species genetic divergence” (Petit et al. 2003).
- On the other hand, there are ecologically transitional regions with high numbers of species, but without a high proportion of endemism (Williams et al. 1999). These are characterised by an overlap of the ranges of species of different

geographical origins caused by dispersal processes along gradients, e.g. the overlap of species belonging to different zonobiomes and azonal communities in the forest-steppe areas of the Carpathian Basin (Varga 1995). Recently, these areas have been identified as “melting pots” of genetic diversity (Petit et al. 2003) due to the secondary accumulation, re-distribution and re-combination of genotypes.

Another important observation was made by de Lattin (1957, 1964) who indirectly defined the “Siberian” species by their absence in the supposed Mediterranean refugial areas. These species show a peculiar “crowding” (de Lattin’s “*Stauungslinie*”) at the Northern boundary of the Mediterranean region. The re-interpretation of this biogeographical line subsequently followed in two steps. First, it was recognised that accumulated occurrence of marginal sub-species of continental species was shown around this line (Varga 1975, 1977), and later on, they were confirmed as multiple extra-Mediterranean refugia of continental species (Schmitt 2007; Schmitt et al. 2007) (Fig. 1).

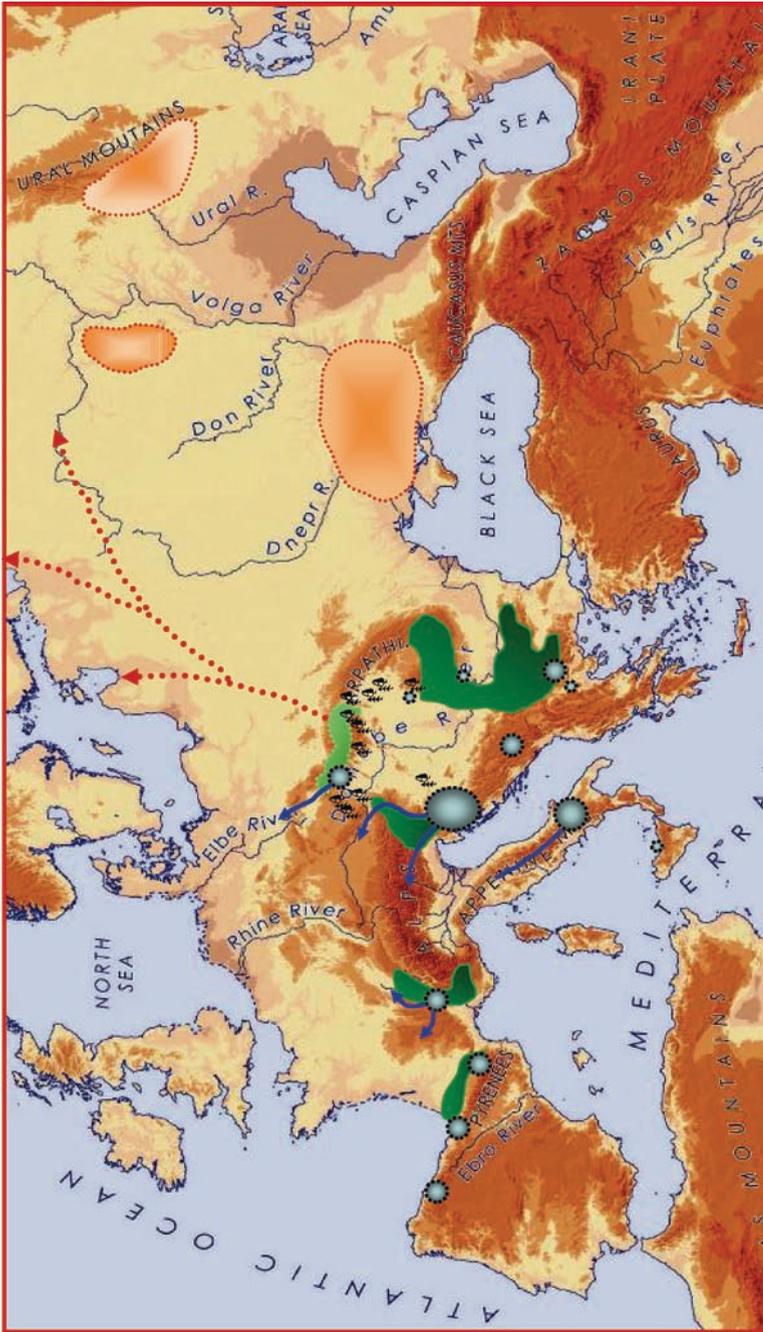


**Fig. 1** Important characteristics of biodiversity in Europe, compiled from various sources. *Purple line*: Southern and Western border of the high (>40%) representation of the Boreo-Continental (= Siberian) faunal elements (de Lattin 1967; Kostrowicki 1969). *Blue line*: Southern border of the expansion (“*Stauungslinie*”) of the Boreo-Continental (= Siberian) faunal elements (de Lattin 1967). *Red dots*: important mountain arboreal refugia (compiled from different sources). *Light blue circles*: core areas of the multispecies genetic divergence of the 25 European forests. *Full circles*: divergence higher than average, *empty circles*: lower than average. *Continuous lines* indicate high divergence, *dotted lines*, intermediate divergence (re-drawn and simplified from Petit et al. 2003). *Green circles*: mean number of haplotypes per forest, averaged across species. Diversity is highest at relatively high latitudes, North of the three European peninsulas (re-drawn and simplified from Petit et al. 2003)

### 1.3 The Geographical Projection of the Genetic Diversity Data

It is not only the number of the species, but also some characteristics of the genetic diversity that show the highest values in the “transitional” belt mentioned above. Based on the chloroplast DNA variation in 22 widespread European trees and shrubs, it was pointed out that despite their “individualistic” migration behaviour, diverse ecological requirements (from the Southern temperate to the Southern boreal zone) and different modes of seed dispersal, significant species of the woody flora of Northern Central Europe exhibited an essentially congruent pattern of genetic divergence (Petit et al. 2003; Fig. 1). Their genetically most unique populations are found in Southern and central Italy, Corsica, and the Balkan Peninsula extending into Northern Italy, the Northern Balkans and the Southern peripheries of the Carpathian Basin. However, this increased diversity is obtained mostly through the redistribution (“melting pot”) of the genetic information already present in the populations in refugia (the actual “hot spots”, i.e. areas where the diversity was evolved). Recent work on the genetics of oak populations has revealed that the existence of particular haplotypes in Romania resulted either from older indigenous relict populations or from crossing of populations originating from more than one refugium. There were several low or medium mountains North of the Balkan Peninsula that could have offered favourable climatic conditions during the Younger Dryas period to support viable populations of oak trees. At the beginning of the post-glacial times, the Carpathian Basin may have acted as a meeting point of several colonisation routes (Bordács et al. 2002; Petit et al. 2002). These genetic data are also supported by recent fossil findings of tree remains from the last full glacial period, North of the Mediterranean peninsulas. Fossil pollen data and macrofossil remains such as charcoals from the time of the last glacial maximum, i.e. 25,000–17,000 years ago, indicate that several tree species remained in small favourable pockets not only within the Mediterranean region, but also at the Southern edge of the cold and dry steppe-tundra area in Eastern, central, and South-Western Europe (Willis et al. 1995, 2000; Carcaillet and Vernet 2001).

Research on small-mammal mtDNA has also questioned the universality of Mediterranean refugia as the areas from which all temperate taxa colonised central and Northern Europe at the beginning of the present interglacial period. It was suggested that the Mediterranean “sanctuaria” (Widmer and Lexer 2001) in general were *not* also core areas of post-glacial expansion into deglaciated areas. Thus, a need for new paradigms for the phylogeography of cold-resistant arboreal species has been formulated, as well (Steward and Lister 2001). It was shown that Mediterranean populations of the shrews *Sorex minutus*, *Sorex araneus* and bank voles (*Chlethrionomys* spp.) did not contribute to the present-day gene pools in Central Europe (Fumagalli et al. 1996; Bilton et al. 1998). Instead, populations in Central Europe and Western Asia have participated suggesting the existence of glacial refugia in these regions. The central European evidences fit well with the botanical results from Hungary and the records of temperate mammals within cold-stage assemblages of central and Northern Europe (Fig. 2).



**Fig. 2** Important extra-Mediterranean refugia, compiled from various sources. *Dark green fields*: European coniferous forest refugia as revealed by Schmitt and Haubrich (2008). *Light green field with brown arrows*: the Northern Carpathian arboreal refugium with expansion routes of boreal species (coniferous trees, brown bear, etc.), from various sources. *Grey circles with blue arrows*: refugia of the beech (*Fagus sylvatica*) with important post-glacial expansion routes (re-drawn from Magri et al 2006; Magri 2007). *Light brown patches*: Eastern European boreal forest refugia during the LGM (from various sources)

## 2 The Basic Types of Zonal Setting in Europe: Glacial Vs. Interglacial

### 2.1 *The Notion of the Boreal Forest-Steppe as the Macro-Ecotone of the Mammoth Steppe and the Boreal Forest*

The Pleistocene glacial-interglacial cycles have resulted in the “*antagonistic dynamics*” of biota belonging to contrasting macrohabitats (de Lattin 1967, p. 356). Thus, principally, two basic types of zonal setting can be distinguished. The inter-/post-glacial (but also interstadial!) type can be characterised by the expansion of the *Arboreal* and regression of the “*non-Arboreal*” macrohabitats. As a result, the “open” macrohabitats (tundra, steppe, semi-desert and high mountains) have been separated by wooded zonobiomes, such as boreal and nemoral forests. Significant belts of macro-ecotones have been developed at the edges of the forested belts, e.g. forest-steppe as a transitional habitat type between the nemoral and steppic zones, subdivided into a sub-mediterranean (Pannonian) and a continental sub-type (Zólyomi 1949; Sjors 1963; Walter and Straka 1970; Varga 1995; Fekete and Varga 2006).

Thus, the glacial periods (stadials) have been characterised by a regression plus fragmentation of wooded habitats and consequently, by a broad contact of the tundra and the steppe zonobiomes with some forested “pockets” North of the refugial belt of the Mediterranean area. The transitional “macro-ecotones” developed at the forest-belt fringes: tundra-taiga and boreal forest-steppe (e.g. in Pannonian region). Recently, cold steppe vegetation with scattered boreal coniferous forests have been revealed during the cold stages in S Moravia, the SE margin of the Alps, E Hungary, parts of Romania (Carpathians), etc. (Rybnicková and Rybnicek 1991; Willis et al. 2000; Pokorný and Jankovská, 2000; Sümegei and Rudner 2001; Wohlfahrt et al. 2001; Björkman et al. 2003; Tantau et al. 2003; Willis and Andel 2004; Feurdean et al. 2007a,b). The presence of these Northern temperate refugia justifies the arguments for the so-called *non-analogue* assemblages of mammals described from the Late Pleistocene in unglaciated areas of the Northern hemisphere.

The Würm glaciation had at least six maxima, the last one taking place about 22.000–18.000 years BP. The Last Glacial Maximum (LGM) was extremely continental and ended in rapid climatic fluctuations. The late Würm *kryoxerotic* period was characterised by the expansion of cold-continental *heliophyta* and steppic species (Iversen 1958; Tarasov et al. 2000; Velichko et al. 2002). Pollen-based “treeless tundra” models for Europe North of the transverse mountain ranges of the Pyrenees, Alps and Carpathians (e.g. Frenzel 1992; Huntley and Birks 1983; Huntley and Allen 2003) have repeatedly been questioned by researchers of the late Pleistocene mammalian fauna (Kretzoi 1977; Guthrie 1990, 2000; Guthrie and van Kolfschoten 1999; Simakova 2001; Yurtsev 2001; Steward and Lister 2001), because the carrying capacity sufficient to feed numerous large herbivores such as the

mammoth, the woolly rhinoceros, the reindeer, the giant deer, the bison or the aurochs demands a very productive environment (“steppe-tundra” or “mammoth steppe”, Guthrie 1990), like the cold-continental meadow steppes in Southern Siberia and Northern Mongolia (Varga et al. 1989).

Although the East-West faunal movements between the Western and Eastern temperate refugia (see: nemoral disjunctions!) were hindered during the last glaciation, the tundra-steppic areas of E Europe and the mountain belts of Central Asia were connected to each other. Thus, *non-analogue communities* were composed by mixing tundra, steppic and eremic-oreal elements (e.g. *Lemmus* and *Dicrostonyx* spp. together with *Citellus* spp. and *Allactaga*, *Sicista*, *Lagurus*, *Marmota* and *Ochotona*). This boreal forest-steppe habitat type appears to have included also cold-tolerant species of temperate habitats, e.g. *Clethrionomys glareolus*, *Apodemus sylvaticus* and *S. araneus*. Extra-Mediterranean core areas have also been revealed in several, widely dispersed cold-tolerant frogs and reptiles, as *Rana arvalis*, *Zootoca vivipara*, *Vipera berus* (Surget-Groba et al. 2001; Babik et al. 2004; Ursenbacher et al. 2006; Kotlík et al. 2006; Saarma et al. 2007).

### 3 European Refugia of Boreal Species

#### 3.1 Biogeographical Definition and Sub-Division of Boreal Species

The species of the boreal zone show a significant diversity of extension and taxonomical structure of ranges. Most boreal taiga-species are widely distributed from the Far East across Siberia to Scandinavia, often without significant geographical variations. They are partly inhabitants of forests but are often connected with peat bogs, e.g. due to the food-plant specialisation (e.g. the butterflies and moths *Colias palaeno*, *Boloria aquilonaris*, *Plebejus optilete*, *Anarta cordigera*, *Arichanna melanaria*). Vicarious taxa often occur in Southern Himalayan coniferous forests with dense scrub layer and undergrowth, rich in evergreen plant species (e.g. *Rhododendron*, *Vaccinium* spp.). Also, some boreal plant species complexes have a huge Holarctic or Eurasiatic range while their closest relatives are restricted to some Southern mountainous areas, as parts of the Himalaya, Tibet, etc., e.g. the boreal species complex consisting of the circum-boreal *Saxifraga hirculus* and the related spot-like Central Asiatic species: *S. diversifolia*, *S. przewalskii* and further species in Tibet. In other cases, the zonal boreal “taiga” distribution is combined with considerable disjunct ranges in the sub-alpine mixed coniferous forests of the Himalayas (e.g. several small passerine birds as *Dumeticola thoracica*, *Luscinia calliope*, *Muscicapa sibirica*, *Phylloscopus proregulus*, *Tarsiger cyanurus*) and/or with several larger or smaller spots in the European mountain coniferous belts, e.g. in many taiga-inhabiting birds, as *Tetrao urogallus*, *Tetrastes bonasia*, *Strix uralensis*, *Aegolius funereus*, *Picoides tridactylus*, etc. (Stegmann 1938; Voous 1963).

Such species are most richly represented in the coniferous zone of the South Siberian high mountains.

The number of (nearly) exclusively European boreal and boreo-montane species is relatively low. However, a molecular biogeographical analysis of such species can unravel the European coniferous forest refugia (Schmitt and Haubrich 2008). The existence of European coniferous forest refugia is also supported by the *Western-Eastern* sub-division of several boreal species with the Western populations obviously having European refugia during the last glaciations, e.g. *Picea abies* most probably in the mountains of the Balkans and in the Eastern and Southern Carpathians. The North-Eastern part of European Russia was populated from the East by the sister (sub-)species *Picea obovata*. In the catchment area of the Northern Dvina, a hybrid belt has been formed between them. The European *Pinus cembra* survived the LGM in Southern Alpine and Carpathian refugia (Willis et al. 2000; Wohlfahrt et al. 2001). Its sister species is the Siberian/Northern Mongolian *Pinus sibirica*, a dominant species of the light-penetrated mountain taiga, often mixed with *Larix sibirica* (Walter and Breckle 1986).

### 3.2 Carpathian–Balkan Boreo-Montane Arboreal Refugia

It is known since several decades that the Southern part of the Carpathian Mts. was a refugial area for temperate and mountain forest taxa during the last glacial period (Huntley and Birks 1983). The Eastern and the Southern Carpathians have been repeatedly pointed out as important glacial refugia, from which trees started to expand at the beginning of the Holocene (e.g. Huntley and Birks 1983; Bennett et al. 1991; Willis 1994). In particular, Willis et al. (2000) indicated that temperate refugia in Europe during cold periods might not have been restricted to the three Southern peninsulas (Iberia, Italy and the Balkans) because trees were undoubtedly present in Central Europe in areas such as the Carpathian Basin during the last cold stage. Radiocarbon data indicate the continuous presence of coniferous woodland not only during the relatively mild period from 35 to 25 kyr BP (thousands of radiocarbon years before the present), but also into the more severe last glacial maximum 25–17 kyr BP (Sümegei and Rudner 2001; Willis and Andel 2004).

The existence of full-glacial forests in Eastern Europe during the LGM (Willis et al. 2000) has often been questioned, but it has also been repeatedly confirmed by several macrofossil and palynological studies from the Carpathians (e.g. Farcaş et al. 1999; Björkman et al. 2003; Tantau et al. 2003; Willis and Andel 2004; Feurdean et al. 2007a) demonstrating the survival of most coniferous and cold-resistant deciduous trees or even the beech (Magri et al. 2006, Magri 2007) in refugial “pockets” in the hilly areas of the Carpathians or North of them. Similarly, forest refugia were also found in Southern Moravia, in the Eastern Alps, in the Dinaric Mountains and in the Eastern Balkans, etc. (Willis 1994; Willis and Niklas 2004).

The climatic conditions in some favourable localities of these regions could not have been as severe as those in Northern and central Europe during the LGM and during the beginning of the last deglaciation.

In the NE Carpathians (Mt Gutaiului), *Pinus mugo*, *P. sylvestris* and later on also *Larix* became established from 14,500 year BP onwards (Wohlfahrt et al. 2001; Björkman et al. 2003). Between 14,150 and 13,950 years BP, *P. cembra* have replaced *P. mugo* and *P. sylvestris*. At 13,950 cal year BP, the tree cover increased and *Picea* appeared for the first time, together with *P. cembra*, *P. mugo* and *Larix decidua*. The analysis of the sediments of the peat bog “Mohos” in the Eastern Carpathians (Tantau et al. 2003) has shown that an open boreal forest was dominated by *Pinus* during the last phase of the LGM. The presence of *Picea* pollen refers to its existence in a local refugium. The pollen data at the end of the late Weichsel indicate that beside the dominant *Pinus* and *Betula* species, *Alnus*, *Ulmus* and *Picea* also occurred in the area. The increased representation of *Alnus* at about 14,150 year BP and that of *Picea* and *Ulmus* between 13,750 and 13,200 years BP is interpreted as a consequence of nearby refugia of these trees. Beech pollen was dated in the North- Eastern Carpathians (Semenic Mts.) to 9,500 year BP while in most other diagrams for the Romanian Carpathians, it was registered between 7,500 and 8,000 years BP. From the late expansion of *Quercus*, *Tilia*, *Fraxinus*, *Acer* and *Corylus* between 10,750 and 10,200 years BP, it was concluded that these trees had to immigrate into the area from refugia further away. However, these refugia cannot have been situated very far from the Carpathians. It seems most likely that these refugia were located further to the South in Romania, or in lower hilly areas of the Carpathian Basin (Feurdean et al. 2007b). Several surveys across Hungary (Rudner and Sümegi 2001; Willis et al. 2001) from about 30 sites clearly demonstrate that cyclically recurring forested habitat developed in the Carpathian Basin during the late Weichselian, although this forest type might have been of an open forest or steppe forest/forest-steppe type. These forests or forest-steppes were of the boreal type mostly with cold-tolerant trees as *Picea*, *Larix*, *Pinus sylvestris* and *P. cembra*, *Betula*, *Carpinus*, *Salix* and *Juniperus communis*.

In Bátorliget, North-Eastern Hungary, a light-penetrated taiga forest and a cold steppe with *Artemisia* spp. dominated during the last cold phase of the Weichselian glaciation. However, it has also been demonstrated that some deciduous trees such as *Quercus*, *Ulmus*, *Alnus* and *Tilia* survived in meso/microclimatically favourable “pockets” of this mosaic landscape (Willis et al. 1995). Later, *Tilia* dominated woodland and a mixture of deciduous trees including *Ulmus*, *Corylus*, *Tilia*, *Fraxinus* and coniferous species (*Pinus* and *Picea*) (Willis et al. 1995, 2000; Magyari et al. 1999) were present in the early Holocene. In a neighbouring area of Romania (Turbuta), pollen of *Quercus* was not found before 12,000 year BP implying that *Quercus* did not survive in the proximity of the study site during the late glacial. Local stands of *Quercus* pollen became established between 12,000 and 11,000 years BP, but its local expansion did not occur until about 11,000 cal year BP and peaked between 10,000 and 8,500 years BP. This is generally simultaneous with the time of *Quercus* expansion in North-Western, Western and South-Western Romania, though in the Eastern Carpathians its expansion was dated to ca.

500 years later (Farcaş et al. 1999, 2004; Björkman et al. 2003; Tantau et al. 2003, 2006; Feurdean and Bennike 2004; Feurdean et al. 2007a).

The fossil and genetic data unanimously indicate that the beech (*Fagus sylvatica*) also survived the last glacial period in multiple refuge areas. Recent publications (Magri et al. 2006; Magri 2007) clearly demonstrate that the central European refugia of the beech were separated from the Mediterranean ones. In addition, the Mediterranean core areas did not contribute to the colonisation of central and Northern Europe. The Illyrian-Slovenian population migrated only to a limited extent Northward to colonise the rest of Europe. This population proved to be genetically similar to the population in Southern Moravia–Southern Bohemia, which is considered to be a possible refuge area on the basis of fossil data. This core area might have been the source for the colonisation of the Carpathians. From a genetic point of view, the populations in the Apuseni Mts. have been characterised by rather high allelic richness (Gömöry et al. 2003). Thus, it is possible that a secondary refugium of the beech was located in this area, which, however, did not significantly contribute to the colonisation of the Carpathian arc.

### ***3.3 Refugia of Cold-Tolerant Invertebrates and Exothermic Vertebrates in Eastern Central Europe***

Vertebrate and Mollusc remains show that the lowest Weichselian loess layers in the Carpathian Basin were formed during the first cool/dry phase of the last glacial, between 50,000 and 70,000 years BP (Hertelendy et al. 1992). The dominance of woodland species decreased, but the tree cover was able to survive the unfavourable environmental changes because, as a result of the mosaic-like environment, some mild and humid micro-climatic areas developed in the foothill zone. On the Tokaj hill, for example, a coniferous forest-steppe was revealed with wildfires in a taiga-like environment during the LGM. Charcoal from *Picea* and podsollic soils were identified (Sümegei and Rudner 2001).

Between 22,000 and 20,000 years BP, a decline of thermophilous gastropod species and expansion of a kryo-xerophilous, *xeromontane* element were observed (Sümegei and Krolopp 2002; Füköh et al. 1995). The occurrence of *Vallonia tenuilabris* was shown together with the boreo–Alpine/montane *Columella columella*. The dominance of the kryophilous land snails reached values of 80% in the Northern parts of the Carpathian Basin while the occurrence of the same group was about 40% in the Southern parts. Data of mollusc analysis refer to a large-scale heterogeneity in both palaeogeographic and palaeobiogeographic conditions for the area examined, creating some sort of a meeting point of faunal elements adapted to different environmental conditions in the region. The “gastropod-thermometer” developed by Krolopp and Sümegei (1995) showed a mean July temperature about 11–12°C in the Northern and 13–14°C in the Southern part of the basin during the LGM. Several further land snails and slugs (*Arianta arbustorum*, *Trochoidea geyeri*, *Arion fuscus* and *Arion* spp.) were also able to survive harsh climatic conditions in

small spots in the central European periglacial (Haase et al. 2003; Pinceel et al. 2005) or even within the Alpine permafrost area. DNA and allozyme data on *A. fuscus* show that its glacial survival was possible in or at the periphery of the Alps as well as in other European mountain ranges (e.g. the Tatra Mountains and Southern Balkans).

In the group of continental species, the woodland ringlet (*E. medusa*) represents a particularly interesting and well-studied case. Good evidences have been found based on allozyme data that this specie had multiple Würm ice-age differentiation centres around the glaciated Alps, in the Carpathian region and in the Balkan Peninsula (Schmitt and Seitz 2001; Schmitt et al. 2007). This glacial distribution pattern of *E. medusa* shows several parallel features with cold-tolerant vertebrates such as the adder, the bank vole, etc. (Deffontaine et al. 2005; Kotlík et al. 2006; Ursenbacher et al. 2006), most probably due to the particular climatic conditions of Eastern Europe during the last ice-ages. Supposedly, the decline of the temperature was, especially in the summer period, less dramatic as in the Atlantic part of Europe. This was combined with a decrease in the precipitation causing a transitional climate and mosaic-like vegetation similar to the recent conditions in the cold-continental forest-steppe belts of Southern Siberia and Northern Mongolia. Three of the four groups of *E. medusa* confined to the Carpathians most probably had their differentiation centres at the low elevations of the Southern-South-eastern Carpathians. The fourth lineage, composed of populations from the Western Carpathians and the North-Eastern Carpathians, might have had a core area of larger extent near to the Northern Carpathians or in the Carpathian Basin. The genetic diversity values of these populations suggest that the differentiation centre in the Carpathian Basin was as large and stable as the ones in the Southern Carpathians (Schmitt et al. 2007). Evidence for Central European refugia was also found for several other woodland species (Steward and Lister 2001). Recently, Schmitt and Haubrich (2008) indicated several Eastern European refugia of the large ringlet (*Erebia euryale*), a butterfly species strictly restricted to the European mountain coniferous forest zone. They concluded that the mountains of South-Eastern Europe were the most important reservoirs of the coniferous forests in Europe.

The Pleistocene glacial refugia of the European *Bombina* toads were located both in the “classical” refugial areas of the Appenins and the Balkans (core areas of *B. pachypus* and sub-species of *B. variegata*, Szymura et al. 2000; Canestrelli et al. 2006) as well as more to the North, in the Carpathians and the adjoining lowlands (Vörös et al. 2006; Hofman et al. 2007). Strong genetic evidences have been provided that *B. variegata* survived the LGM in the Carpathians. The mtDNA and allozyme data suggest two separate refugia. One clade probably had its refugium in the Southeastern edge of the Carpathians while the most likely refugium of the other clade was in the Southern Carpathians, where the haplotype diversity is the highest. However, the deep genetic divergence among European *Bombina* lineages suggests their pre-glacial origin.

The European populations of the moor frog are subdivided into three genetic lineages from which two are exclusively found in the Carpathian Basin. They, most probably, survived the LGM in the Carpathian Basin and have expanded to the

North to a rather limited extent only (Babik et al. 2004). The survival of *R. arvalis* in the Carpathian Basin was also demonstrated with fossil records (Venczel 1997). This view is supported by further recent data suggesting that this region might have been an important LGM refugial area in other amphibians and reptiles, as well. In the lizard *Z. vivipara*, a haplotype restricted to Northern Hungary and Austria was found in a species-wide survey of mtDNA variation (Surget-Groba et al. 2001). This ovoviviparous form shows peculiar karyotypic characters and demonstrates that the ovovivipary independently evolved in the Western European and Pannonian (*Z. vivipara pannonica*) populations (Kupriyanova et al. 2006; Surget-Groba et al. 2006). The suggested refugia of the adder (*V. berus*) would be in the Southern pre-Alpine lowland (Italian clade), in the “Illyrian” core area in the Western Balkan peninsula (Balkan clade) and several core areas of the Northern clade: in the Carpathian region (Carpathian sub-clade), East of the Carpathians (Eastern subclades) and in a location between Great Britain and Poland (Central European subclade). The survival of two mitochondrial lineages in the Carpathian Basin throughout the LGM was postulated in this species, one East of the Alps, and the other in the North-Eastern part of the basin or even in the Eastern Carpathians (Carlsson 2003; Ursenbacher et al. 2006).

### **3.4 Refugia of Boreal Birds and Mammals in Eastern Central Europe**

Comparison of phylogeographic structures in several Eurasiatic boreal species has shown that species associated with the taiga forest revealed essentially similar patterns. In the wood lemming (*Myopus schisticolor*), and also in most other boreal forest species, no substantial phylogeographic divisions across Northern Eurasia have been reported (Zink et al. 2002; Fedorov et al. 2008). The contraction of the range of these species to a single, probably Southern Siberian refugial area during the late Pleistocene followed by demographic expansion seems to be a general background for their shallow phylogeographic structure. The most important genetic discontinuity has usually been observed between the “Northern” Eurasiatic and the “Far East” clades. The limited distribution range of the South-eastern lineages suggests that their core areas (“Manchurian refugium” of de Lattin 1967) could not play an essential role in the post-glacial colonisation of Northern Eurasia by boreal forest species. A weak phylogeographic structure was also discovered in the flying squirrel (*Pteromys volans*), in which the divergence between the Far Eastern and Northern Eurasian groups may have been initiated during the Weichselian glaciation (Oshida et al. 2005). Mountain ranges in Southern Siberia and North-Eastern China may have isolated the Far Eastern group. Although the largest part of North-Eastern Europe and Siberia was not covered with ice during the last glaciation, most parts were overgrown by cold tundra-steppe (“mammoth steppe”) under extremely cold and dry conditions (Svendsen et al. 1999, 2004; Simakova 2001; Schirrmeister et al. 2002). At that time, multiple isolated refugia of *P. volans* could be formed in

Eurasia. After the last glaciation, *P. volans* might have expanded throughout Northern Eurasia, along both sides of the Ural mountains.

Other boreal species show clear phylogeographical structures with well-differentiated populations in the coniferous forest zone of the South European mountains. They belong to rather different taxonomical groups. The brown bear complex can be mentioned as a classical example, (e.g. Taberlet and Bouvet 1994; Valdiosera et al. 2007). The discovery of 18,000-year-old charcoal of yew (*Taxus baccata*) and Scots pine (*P. sylvestris*) in Western Slovakia (Litynska-Zajac 1995) indicates that the climatic conditions at the Western Carpathians might have been suitable for the brown bear. The Eastern Carpathians served also as a refuge area for brown bears during the last glacial phase. Nevertheless, recolonization from the Eastern Carpathian refuge appears to have been less effective compared to the migration that began from the North-Western Carpathian refuge. This recolonization pattern can be explained by the more Northern position of the North-Western Carpathians (leading edge).

Southern European montane refugia have also been revealed in the capercaillie (*T. urogallus*). It was suggested that the Southern European *aquitanus* lineage had expanded throughout Europe before the LGM, and the Eastern, *urogallus* lineage expanded in Asia and North-Eastern Europe. During the LGM, the two lineages were restricted into separate refugia (*aquitanus*: Iberia and Balkans, *urogallus*: Southern Siberia). During the post-glacial re-forestation, the *urogallus* lineage replaced *aquitanus* in Europe and forced them to the South-west into their refugia in the Pyrenees and Cantabrian Mts. (Duriez et al. 2007).

Different phylogeographical structures have been revealed in the root voles (Brunhoff et al. 2003), lemmings (Fedorov et al. 1999), collared lemmings (Fedorov and Stenseth 2002), and common voles (Haynes et al. 2003; Fink et al. 2004). In these cases, the Ural Mountains separated the Northern European and Siberian lineages. Root voles in Europe form a Northern and a central mtDNA phylogroup. Fossil records from the last glaciation have demonstrated that collared lemmings (*Dicrostonyx*) and true lemmings (*Lemmus*) used to be the most common small mammals in the periglacial tundra-steppe of central Europe. These species were regularly accompanied by several vole species, including the root vole. These species assembly always remained North of the “classical” Southern European refugia. Thus, central European root vole populations can be considered the sources of Northward expansion during the deglaciation. This model is corroborated by the data demonstrating that the fragmented populations in the Netherlands, Slovakia and Hungary all belong to a single mtDNA phylogroup. Consequently, the threatened populations in Hungary, Austria, Slovakia and the Netherlands represent glacial and post-glacial relicts (van de Zande et al. 2000).

Bilton et al. (1998) show that the Mediterranean populations of *S. minutus*, *S. araneus* and *Clethrionomys* spp. did not contribute to the present-day gene pools of the Central and Northern European populations. This view has also been supported by the high rate of endemism in the Mediterranean bank vole phylogroups. In contrast with some forest rodents (*A. sylvaticus*, *A. flavicollis*), bank voles would not be restricted to

the Southern forest refugia during the last glaciations. Therefore, bank vole populations might have survived the Quaternary glaciations in their Northern refugia. This may have resulted in their present complex phylogeographic pattern including multiple glacial refugia in central Europe, in the Mediterranean mountains and possibly also in several Eastern regions. Based on fossil records, the Carpathian region has been suggested as a glacial refugia area for this species. These refugia were most likely to be located near the Alps or in the Carpathians and possibly at the network of streams in the marginal areas of the Carpathian Basin. Phylogeographic analyses of the bank vole have already suggested that glacial refugia located in central and Eastern Europe made a major contribution to the modern population of this species in Europe (Deffontaine et al. 2005; Kotlík et al. 2006). The analysis of nucleotide diversity also demonstrates that these Eastern Central European regions acted as core areas of expansion for the Western lineage of the bank vole. Another refugial area was proposed in the regions of Eastern Romania, Western Azov Sea and the Crimea (Jaarola and Searle 2002).

## **4 Area Dynamics, Evolution and Diversity Patterns in the Carpathian Basin**

### ***4.1 Endemic Taxa and Autochthonous Evolution in the Carpathian Basin***

The level of endemism generally correlates with the geological age of the refugia where relict-like taxa have been evolved and/or could survive. The Carpathian Basin belongs to the geologically young areas of Europe. Its relief developed under the influence of the Alpine orogenesis and by retreat of the Paratethys and the Pannonian inland sea. Moreover, the phylogeography of some freshwater invertebrates (e.g. Neritidae snails, see: Bunje 2007; Fehér et al. 2007) is clearly connected with the evolution of the Ponto-Pannonian water basin and of the Danube catchment area. In addition, there are several taxonomical groups with considerable proportion of endemic species, e.g. the land gastropods (Soós 1943) the earthworms (Lumbricidae: Csuzdi and Pop 2007) or some soil arthropods (e.g. Opiliones, Diplopoda: Korsós 1994; Collembola: Dányi and Traser 2007). Their core areas clearly coincide with the younger tertiary land masses within and near the Carpathian Basin.

Most endemic species are narrow specialists, inhabiting extreme habitats, such as thermal springs, karstic caves and karstic springs (Table 1). Several endemic troglobionta have been described in gastropods, pseudo-scorpions, harvestmen, spiders and springtails, often occurring within a single or a few caves of karstic mountains. Several species of earthworms, millipedes, centipedes and assels can be considered as holo-endemic species of the Western Transylvanian (Apuseni) mountains (Csuzdi and Pop 2007; Varga and Rakosy 2008).

Endemic terrestrial insects of the Carpathians are, as a rule, short-winged, flightless species such as the bush-crickets *Isophya*, *Poecilimon* spp.; some stenotopic relict grasshoppers (*Capraiuscola ebneri*, *Podismopsis transsylvanica*, *Uvarovitettix transsylvanica*, *Zubovskia banatica*; Kis 1965, 1980); numerous species of the

**Table 1** Examples of stenotopic species of extreme habitats endemic to the Carpathians and the Carpathian basin

Taxonomic group	Species	Habitat type	Occurrence
Gastropoda	<i>Melanopsis parreysi</i>	Thermal springs	Baile Felix (Oradea)
Gastropoda	<i>Theodoxus prevostianus</i>	Thermal springs	Hungarian Middle Range
Gastropoda:	<i>Paladilhia hungarica</i>	Karstic water in caves	Mecsek Mts. (Abaliget)
Gastropoda	<i>Paladilhioipsis transsylvanica</i>	Karstic water in caves	Mți. Apuseni
Gastropoda:	<i>Paladilhioipsis leruthi</i>	Karstic water in caves	Mți. Apuseni
Amphipoda:	<i>Niphargus tatrensis</i>	Karstic water in caves	Calcareous mts. in the Northern Carpathians
Gastropoda	<i>Bythinella (Sadleriana) pannonica</i>	Karstic springs	Calcareous mts. in the Northern Carpathians
Palpigradi:	<i>Eukoenia vagvoelgyii</i>	Karstic caves	Aggtelek karst, Slovakian karst
Araneae	<i>Centromerus chappuisi</i>	Karstic caves	Mți. Apuseni
Araneae	<i>Troglohyantes racovitzai</i>	Karstic caves	Mți. Apuseni
Araneae	<i>Nesticus biroi</i>	Karstic caves	Mți. Apuseni
Diplopoda	<i>Haasea hungarica</i>	Karstic caves	Mecsek Mts. (Abaliget)
Diplopoda	<i>Hungarosoma bokori</i>	Karstic caves	Mecsek Mts. (Abaliget)
Isopoda	<i>Mesoniscus graniger</i>	Karstic caves	Aggtelek karst, Slovakian karst
Collembola	<i>Pumilnura dudichi</i>	Karstic caves	Aggtelek karst
Collembola	<i>Protaphorura kadici</i>	Karstic caves	Aggtelek karst
Collembola	<i>Arrhopalites dudichi</i>	Karstic caves	Aggtelek karst
Collembola	<i>Arrhopalites hungaricus</i>	Karstic caves	Aggtelek karst
Collembola	<i>Arrhopalites bueckensis</i>	Karstic caves	Bükk Mts. (N. Hungary)
Coleoptera, Carabidae	<i>Duvalius bokori</i>	Karstic caves	Slovakian karst
Coleoptera, Carabidae	<i>Duvalius gebhardtii</i>	Karstic caves	Bükk Mts.
Coleoptera, Carabidae	<i>Duvalius microphthalmus</i>	Karstic caves	Calcareous mts. in the Northern Carpathians
Coleoptera, Carabidae	<i>Duvalius hungaricus</i>	Karstic caves	Aggtelek karst, Slovakian karst
Coleoptera, Carabidae	<i>Duvalius redtenbacheri</i>	Karstic caves	Mți. Apuseni
Coleoptera, Carabidae	<i>Duvalius biroi</i>	Karstic caves	Mți. Apuseni

**Table 2** Examples of endemic species of forest soils in Transylvania

Lumbricida	<i>Allolobophora dacica</i>	Forest soil + litter	Mți. Apuseni
Lumbricida	<i>Octodrilus</i> spp. (12 spp.!)	Forest soil + litter	Mți. Apuseni
Pseudoscorpiones	<i>Neobisium brevipes</i>	Forest soil + litter	Mți. Apuseni
Opiliones	<i>Ischyropsalis dacica</i>	Forest soil + litter	Mți. Apuseni
Diplopoda	<i>Trachysphaera biharica</i>	Forest soil + litter	Mți. Apuseni
Diplopoda	<i>Typhloiulus serbani</i>	Forest soil + litter	Mți. Apuseni
Myriopoda	<i>Monotarsobius paucioculatus</i>	Forest soil + litter	Mți. Apuseni
Myriopoda	<i>Monotarsobius spelaeus</i>	Forest soil + litter	Mți. Apuseni
Myriopoda	<i>Monotarsobius burzenlandicus</i>	Forest soil + litter	Mți. Apuseni
Myriopoda	<i>Lithobius dacicus</i>	Forest soil + litter	Mți. Apuseni
Isopoda	<i>Cylisticus major</i>	Forest soil + litter	Mți. Apuseni
Isopoda	<i>Trachelipus spinulatus</i>	Forest soil + litter	Mți. Apuseni
Isopoda	<i>Biharoniscus racovitzai</i>	Forest soil + litter	Mți. Apuseni

ground beetles (*Duvalius*, *Trechus*, *Patrobus*, *Morphocarabus* spp.) and weevils (e.g. *Otiorrhynchus* spp.). A bulk of these endemic taxa is confined to the Eastern and Southern Carpathians, to the Apuseni Mts. and to the mountains of Banat, which could preserve relict species (e.g. the tertiary relict gastropods *Chilostoma banaticum*, *Pomatias rivulare*) or some narrow endemic species of Isopoda and Diplopoda (Table 2) in refugia without permafrost phenomena during the last glaciations (Bennett et al. 1991; Krolopp and Sümegi 1995; Willis et al. 1995).

In the more mobile insect groups, the proportion of endemism lies rather low (e.g. in *Odonata* no endemic taxa occur in the Carpathian Basin). Most endemic Lepidoptera of the Carpathian Basin belong to Microlepidoptera, which have flightless females and are strictly specialised to some food plants living on halophyta in the saline grasslands of the Fertő-Neusiedlersee area (Kasy 1965) and those of the Great Hungarian plain (Kiskunság and Hortobágy). Endemic subspecies of *Geometridae* and *Noctuidae* evolved as peripheric isolates of turano-eremic species from the late-glacial, *kryoxerotic* periods, e.g. *Narraga tessularia kasyi*, *Saragossa porosa kenderesensis* (on food plants: *Artemisia santonicum*, *A. pontica*) and *Hadula dianthi hungarica* (on *Gypsophila muralis*). Some endemic taxa in the sandy areas of the Pannonian lowland are specialised predators or parasitoids, e.g. the spider *Dictyna szaboï* and the pompilid wasp *Cryptocheilus szabopatayi*. Further species described as endemics later proved widely-dispersed steppicolous species. In their majority, the endemics of the lower, hilly parts of the Carpathian Basin, however, represent thermophilous post-(inter?-) glacial relicts with connections to the Balkan Peninsula, Asia Minor or Southern Russia (e.g. *Apamea sicula tallosi* in warm-humid alluvial areas, *Dioszeghyana schmidtii schmidtii* and *Asteroscopus syriacus decipulae* in Pannonian xerothermic oak forests, *Polymixis rufocincta isolata* in the Villányi Mts.; *Chersotis fimbriola fimbriola*, *Euxoa vitta vitta*, *E. hastifera pomazensis* and *Cucullia mixta lorica* in the dolomitic areas of the Transdanubian Middle Range, *Chersotis fimbriola baloghi* in the Aggtelek Karst). Balkanic connections have also been observed in butterfly species, which

are restricted to special, Pontic-Pannonian steppic food plants, e.g. *Plebeius sephirus* (feeding on *Astragalus exscapus*, *A. dasyanthus*), *Melitaea telona kovacsi* (on *Cirsium pannonicum*).

Several endemic Macrolepidoptera subspecies of the Carpathians belong to the genera *Erebia* and *Glacies* (Table 3). A few endemic taxa are only widespread in the Carpathians and in the neighbouring mountainous areas, e.g. *Aricia artaxerxes issekutzi*, *Photedes captiuncula delattini*, while others are confined to the Southern Carpathians, often with Balkanic connections: *Erebia cassioides neleus*, *Coenonympha rhodopensis schmidtii* (Varga 1975, 2003a). The subspecies of *Erebia melas* inhabit island-like, calcareous mountain stocks, *E. melas runcensis* in the Apuseni Mts., *E. melas melas* in the Cernei Mts. and *E. melas carpathicola* in the Eastern Carpathians). All these data clearly demonstrate that the Carpathians, especially the Eastern and Southern parts, together with the mountains of Western Transylvania (Apuseni Mts. and Banat) can be considered as core areas for the survival and autochthonous evolution in many invertebrate groups of limited mobility.

## 4.2 The Carpathian–Balkan Connections

The close geological and faunal connections of the Carpathians suggest the existence of highly dynamic contacts with the mountains of the Balkan Peninsula during the Upper Pleistocene. These connections show a contrasting picture compared to the refugia of the Iberian and the Apennine peninsula which have been much more sheltered by the glaciated mountains of the Pyrenées and the Alps, respectively. At least two major arboreal refugia can be traced here: the Illyrian refugium related to the Dinarids and its foothills and the Carpatho-Dacian refugium related to the Carpathians and its foothills. Some areas attached to these refuges served as periodic habitats over climatically favourable periods. These are regarded as fluctuation zones (Varga 1995; Sümegi et al. 1998; Deli and Sümegi 1999).

Since the Carpathian Basin occupied a transitional position between the Balkanic refugia and the cold-continental tundra-steppe zone during the glacial periods, the post-glacial re-population of the Carpathian Basin proceeded (1) by long-distance dispersal from the more remote (atlanto- and ponto-) Mediterranean and Southern Continental refugia, and (2) also from some adjacent local survival areas, e.g. from North-Western Balkanic (“Illyrian”) versus South Transylvanian (“Dacian”) arboreal refugia. In such cases, the arrows of the Northwards dispersal of the South-Western and South-Eastern populations surround the arid central part of the basin. These components of the flora and fauna extend Northwards through the foothills of the Eastern Alps and Southwest-Pannonian hilly regions on the one hand, and through the hilly regions of the Banat area and the Western foothills of the Transylvanian “Island” mountains (Apuseni Mts.), on the other. In some cases, the populations of the South-Western and South-Eastern “strains” do not display any significant taxonomical differentiation, e.g. the silver lime (*Tilia tomentosa*) or

**Table 3** Endemic subspecies of *Erebia* (Nymphalidae, Satyrinae) and *Glacies* (Geometridae, Ennominae) in the Carpathians (maps of *Erebia* spp.: Varga 1975; Varga and Schmitt 2008)

Name	Typical locality	Range
<i>Erebia manto traianus</i> (Hormuzaki, 1895)	Rareu (NE Carpathians)	NE-SE-S Carpathians
<i>Erebia epiphron transsylvanica</i> (Rebel, 1908)	Bucegi (SE Carpathians)	the whole Carpathian arc
<i>Erebia pharte belaensis</i> v. Goltz, 1937 (= <i>E. pharte</i> <i>carpatina</i> A. Popescu-Gorj & A. Szabó, 1986)	“Belaer Kalkalpen” (= Belanské Tatry) Rodna Mts. (NE Carpathians)	N + E Carpathians, S Carpathians
<i>Erebia sudetica radnaensis</i> (Rebel, 1915)	Rodna Mts. (NE Carpathians)	NE Carpathians, S Carpathians
<i>Erebia gorge rudkowskii</i> (Bang-Haas, 1933)	Tatra Mts.	Tatra Mts., NE Carpathians
<i>Erebia gorge fridericikoenigi</i> (Varga 1998)	Piatra Craiului (SE Carpathians)	SE-S Carpathians
<i>Erebia cassioides neleus</i> (Freyer, 1833)	“Banater Gebirge”	S Carpathians
<i>Erebia melas melas</i> (Herbst, 1796)	“Transylvania” (Mți Cernei)	Mți Cernei (Domogled)
<i>Erebia melas carpathicola</i> (Alexinschi and Popescu- Gorj, 1959)	Haghmașul Mare (E Carpathians)	E-SE Carpathians
<i>Erebia melas runcensis</i> (König, 1965)	Cheile Runcului (Mți Apuseni)	Mți Apuseni
<i>Erebia pronoë regalis</i> (Hormuzaki, 1937)	Bucegi (SE Carpathians)	SE Carpathians
<i>Erebia pandrose roberti</i> (Peschke, 1920).	Tatra Mts.	Tatra Mts, NE Carpathians
<i>Erebia pandrose cibiniaca</i> (Dannehl, 1927)	“Cibinsgebirge”	SE-S Carpathians
<i>Glacies canaliculata</i> <i>schwingenschussi</i> (Wehrli, 1921)	Tatra Mts.	higher mountains of the whole Carpathian arc
<i>Glacies noricana carpathica</i> (Schwingenschuss, 1915)	“Belaer Kalkalpen” (= Belanské Tatry), Mt. Bujacie	Belanské Tatry, Bucegi, Retezat
<i>Glacies bentelii retyezatensis</i> (Bartha, 1933) – subspecific status dubious!	Retezat Mts. (Lac. Zenoaga)	Retezat, Bucegi (ssp.?)
<i>Glacies coracina dioszeghyi</i> (Schmidt, 1930)	Retezat Mts. (Lac. Zenoaga)	Retezat, Bucegi (ssp.?)

some butterflies and moths (*Pyronia tithonus*, *Aplasta ononaria*, *Idaea nitidata*, *Zanclognatha tenuialis*). Much more evidence is provided by the re-population of the Carpathian Basin from different directions in the cases of vicarious pairs of closely related species or in subspecies of polycentric species. Such cases can mostly be mentioned in land gastropods, e.g. *Pomatias elegans* – *P. rivulare*,

*Chilostoma illyricum* – *Ch. banaticum*, or in flightless insects, e.g. short-winged Orthoptera: *Odontopodisma schmidti* – *O. rubripes*, *Isophya modestior* – *I. stysi* (Orci et al. 2005).

The Western Balkanic (“Illyrean”) influences are most significant in the Southern and South-Western parts of Transdanubia. These areas are characterised by a humid sub-Mediterranean climate and do not have a significant rainfall deficit in the summer period. They belong to the belt of mesophilous zonal forests of *Fagion illyricum* and *Quercus-Carpinion illyricum* and the Illyrean-Pannonian hardwood gallery forests (*Fraxino pannonicae-Ulmetum*) characterised by a richness in tertiary/inter-glacial relict, often geophytic plant species (Horvat et al. 1974).

The Transylvanian (“Dacian”) influences are connected with the forested areas of the Eastern Carpathians and often transmitted by the Western Transylvanian mountains (Mahunka 1993, 2007; Varga 1989, 1995, 2003a,b). The occurrence of Dacian elements is typical of the Eastern part of the Hungarian Middle Range, especially in the higher parts of the volcanic Eperjes-Tokaj range and in the Karst areas of N Hungary and S Slovakia. Eastern Balkanic influences reach also the Hungarian Middle Range by relict-like occurrences of some Balkanic and Balkanic-Anatolian elements (e.g. *Noctuidae*: *A. syriacus* and *D. schmidtii*), especially in the warm foothill zone where the sub-Mediterranean influences are also significant. Relict occurrences of Dacian elements (bush-crickets: *Isophya stysi*, *Leptophyes discoidalis*, *Pholidoptera transsylvanica*; ground-beetles: *Carabus hampei ormayi*) have been recently discovered on the small, island-like volcanic hills of the Bereg lowland.

The influences of the Northern Carpathians are also significant in the NE part of the Hungarian Middle Range. There is a characteristic difference between the Eperjes-Tokaj volcanic chain on the one hand, and the limestone plateau of the Bükk Mts. and the N Hungarian karst on the other. The biotic contact of the Eperjes-Tokaj range with the Carpathians is young, obviously post-glacial, and can be characterised mostly by the presence of species, which are either typical of the montane forest belts of the Carpathians (e.g. numerous land snails: *Bielzia coeruleans*, *Vestia gulo* and ground-beetles: *Carabus obsoletus*, *C. zawadzkyi*, *Abax schueppeli*) or widely dispersed in the Northern part of Central Europe, often having a Euro-Siberian distribution. The Bükk Mts., however, display an insular character. Its Carpathian and de-Alpine elements (e.g. land snails: *Spelaediscus triaria*, *Phenacolimax annularis*, the Geometrid moth *Entephria cyanata gerennae*) are isolated relicts. In the Aggtelek Karst area, the immediate contact with the higher limestone plateaus of Slovakia is combined with the occurrence of Carpathian (land snails: *B. coeruleans*, *Cochlodina cerata*, *Trichia unidentata*; ground-beetles: *C. obsoletus*, *C. zawadzkyi*, *A. schueppeli*, *Trichotichnus laevicollis carpathicus*), boreal and xeromontane species at surprisingly low altitudes, influenced by the conspicuous meso-climatic and geomorphological features of this area. Some influences of the Northern and the Eastern Carpathians are to be observed at the NE marginal areas of the Pannonian lowland, i.e. along the upper course of the river Tisza and its tributaries (e.g. occurrence of land gastropods *Vitrea diaphana*, *B. coeruleans*, *Balea stabilis*, *Perforatella dibothrion*, *P. vicina*).

### 4.3 *The Forest: Steppe Dynamics in the Carpathian Basin*

The Carpathian Basin belongs to the regions of Europe with the highest biodiversity (Williams et al. 1999). Due to its transitional position during the Quaternary climatic fluctuations, the overlap and accumulation of floristic and faunistic elements of contrasting habitats occurred here. This overlapping of different climatic provinces, enhanced by the varied relief, edaphic and hydrographic conditions, has resulted in suitable conditions for the survival of a large number of species belonging to different core areas and displaying various patterns of long-distance and short-distance re-populations.

It is typical for South-Eastern Central Europe that the large-scaled zonal settling of vegetation, characteristically developed in the East-European table-land, breaks down. In the Carpathian Basin, the concentric arrangement of vegetation belts is influenced by numerous climatic, orographic, hydrographic and edaphic factors (Varga 1995, 2003b). The forest-steppe, which is typical in the major, central lowland and hilly parts of the basin, is represented by a number of regional variants showing distinct geological, edaphic and meso-climatic characters. The forest, skirt and grassland compartments of each regional variant of the forest-steppe are highly intercorrelated. The Carpathians transmit (e.g. boreal) also filter certain different (e.g. steppic) biogeographical influences. Populations passing through the Carpathians will often be isolated and differentiated from the populations inhabiting other parts of their range of distribution.

The geographically transitional position of the Carpathian Basin resulted in a conspicuous mixture of faunal elements of diverse origins and geographical histories. The compartment structure of the vegetation complexes, typical for the Pannonian forest-steppe, has promoted the survival of very different faunal elements. Especially, the hilly areas of transitional climatic conditions surrounding the Pannonian lowland are populated by numerous, biogeographically important species and communities. The Southern, xerothermic slopes and foothills of the Hungarian Middle Range served both as refuges for thermo-xerophilous elements during several cold and cool-humid climatic phases of the Quaternary and as centres of their dispersal (Soó 1940, 1959; Wendelberger 1954, 1959; Zólyomi 1949, 1953, 1964). Thus, many thermophilous elements probably populated the Carpathian Basin not only by long-distance colonisation from remote, large glacial refuges, but also from numerous meso- or microclimatically favourable sites lying at the fluctuating borderlines of the Mediterranean refugial and periglacial belts. The varied and fine biostratigraphical structure of the Hungarian young Pleistocene, often characterised by a coexistence of forest and non-forest faunal elements (e.g. Jánossy 1979; Kordos 1977; Kretzoi 1969, 1977), provides evidences to support this view and demonstrates the transitional biogeographical character of this region during the whole time-span of the Quaternary period. New palynological data from the Eastern part of the Pannonian lowland (Bátorliget) also suggest the presence of forest refuges during the last glacial period (see also: 3.2.).

#### 4.4 *Relict Species with Long-Distance Disjunctions in the Carpathian Basin*

There are several Mediterranean-Manchurian bicentric faunal elements with disjunct range occurring in the Carpathian Basin. The distribution of this species group is connected with the Ponto-Caspian waterway-system, and displays long-distance disjunctions from the vicarious Eastern Asiatic taxa, which often are only subspecifically differentiated (Lepidoptera: *Apatura metis metis* – *Apatura m. substituta*, *Chariaspilates formosarius hungaricus* – *Chariaspilates f. formosarius*, *Rhyparioides m. metelkanus* – *Rhyparioides metelkanus flavidus*, *Arytrura musculus* ssp. – *Arytrura m. musculus*). These and also some other species of this group (*Polygogon gryphalis*, *Herminia tenuialis*, *Diachrysia nadeja*) occur at the lower course of the Danube and the Drava as well as in swampy-boggy areas of the lowlands in Transdanubia, in the Banat and Eastern Hungary. The refugia of these faunal elements were probably along the lower courses of the Danube and its tributaries. Gallery forests of the Illyrian and the Pannonian type and alluvial wetlands accompanying the large rivers of the Pannonian lowland served as corridors for the Northwards expansion of these species.

Different types of long-distance disjunctions have been observed in the relict-like steppe and semi-desert species. The polytypic butterfly *Melanargia russiae*, which is widespread in West and Central Asia, South Siberia and in the mountains of Italy and the Balkan Peninsula, occurred locally – as *M. russiae clotho* – on tall-grass clearings of birch gallery forests of the sandy lowland in Kiskunság. Its extinction was partly due to the consequence of overcollecting, and mostly because of destroying the habitats (re-forestation with *Robinia pseudoacacia*). The habitats of *Chondrosoma fiduciarium* (Kasy 1965) are also tall-grass lowland and hilly steppes often mixed with slightly saline patches. Other species are confined to open dolomitic rocky swards (e.g. *Phyllometra culminaria*, *Lignyopectera fumidaria*, *C. mixta lorica*) or open sandy and rupicolous grasslands (*Oxytripia orbiculosa*, vanishing).

Eremic species are restricted to semi-desert-like habitats of the lowland with extreme edaphic conditions. This faunal type is represented by very few vertebrates: only the small rodent *Sicista subtilis* and the short-toed lark *Calandrella brachydactyla* belong to this group. Abundant examples can be found in strictly localised phytophagous insects, which are often connected with special halophytic plant communities. They are often represented by endemic Pannonian subspecies or allopatric sibling species of Turanian origin, e.g. the Noctuid moths *Saragossa porosa kenderesiensis* and *H. dianthi hungarica* or the Microlepidoptera: *Coleophora hungariae*, *C. klimeschiella*, *C. magyarica*, *C. peisoniella*, *Holcophora statices*, *Stenodes coenosana*, *Agriphila tersella hungarica*, etc. The dispersal of this species group could have taken place in the late glacial phases on the Pannonian lowland, with a subsequent isolation as a result of the post-glacial expansion of the forested belts.

Last but not the least, *xeromontane* elements are present also in the Carpathian Basin. Their two main groups are: the Mediterranean-xeromontane species,

represented by a few vertebrates (e.g. *Monticola saxatilis* or the secondarily more expanded *Phoenicurus ochrurus*). A larger number of species, however, belongs to some insect groups, e.g. Noctuidae (*E. vitta*, *E. decora*, *E. birivia*, *Dichagyris candelisequa*, *Yigoga nigrescens*, *Chersotis margaritacea*, *Ch. fimbriola*, *Apamea platinea*, etc.) and Orthoptera (e.g. *Paracaloptenus caloptenoides*). The continental-xeromontane type is represented by some members of widely distributed Asiatic mountain steppe species as *Euxoa recussa*, *Dichagyris musiva* (Noctuidae) and by some relict-like inhabitants of the rocky dolomitic grasslands as *P. culminaria*, *L. fumidaria* (Geometridae). It seems to be very probable that numerous genera, typical for the steppe biome, might have a xeromontane origin (especially Lycaenidae: e.g. the subgenus *Agrodiaetus* and other species groups of *Polyommatus* and *Plebeius*; Satyridae: *Chazara*, *Pseudochazara*, *Hyponephele*; Noctuidae: *Euxoa*, *Agrotis*, *Dichagyris*, *Yigoga*, *Rhyacia*, *Chersotis*, *Eugnorisma*, etc.). The same has to be supposed in the case of some endemic elements of the Pannonian flora (*Linum dolomiticum*, *Seseli leucospermum*, *Ferula sadleriana*, *Onosma tornense*, etc.).

## 5 Summary and Conclusions

The European fauna has traditionally been subdivided into a “*holothermic*” refugial and a “*holopsychric*” invasion type. The former type was differentiated according to the secondary subdivision of the Mediterranean refugial area. This view was confirmed and modulated by molecular results. A general conclusion was that temperate species mainly derive from Mediterranean refugial populations that underwent range expansion in the late glacial and early post-glacial periods. The other main group has been considered for a long time as a result of the “Siberian” invasion despite the evidences, which have revealed the taxonomical differentiation of North-Eastern “boreal” and Southern European montane populations. Several authors have suggested an additional mode of colonisation of Central and Northern Europe by *non-Mediterranean* populations, coming from one or more “continental” refugia. Fossil pollen data and macrofossil remains from the time of the Last Glacial Maximum indicate that several tree species remained in small favourable spots at the Southern edge of the steppe-tundra area. Research on small mammals has also questioned the universality of Mediterranean refugia. It was suggested that the Mediterranean “sanctuarium” in general were *not* also core areas of post-glacial expansion into deglaciated areas.

The Pleistocene glacial-interglacial cycles have resulted in the “*antagonistic dynamics*” of biota pertaining to contrasting macrohabitats. Principally, two basic types of zonal setting can be distinguished. The glacial periods have been characterised by a regressive fragmentation of wooded habitats and, consequently, by a broad contact of the tundra and the steppe zoniomes with some forested “pockets” North of the refugial belt of the Mediterranean area. Transitional *zono-ecotones* developed at the forest-belt fringes: tundra-taiga and boreal forest-steppe. Pollen-based

“tree-less tundra” models for Europe, North of the transverse mountain ranges, have repeatedly been questioned by researchers of the late Pleistocene mammalian fauna because the carrying capacity sufficient to feed numerous large herbivores demands a very productive environment (“mammoth steppe”). Thus, non-analogue communities were composed by mixing tundra, steppic and eremic-oreal elements. This boreal forest-steppe habitat type appears to have included also cold-tolerant species of temperate habitats.

The species of the boreal zone show a significant diversity of extension and of taxonomical structure of ranges. Comparison of phylogeographic structures in several Eurasiatic boreal species has shown that species associated with the taiga forest revealed essentially similar patterns. The number of exclusively European boreal and boreo-montane species is relatively low. However, a molecular biogeographical analysis of such species can unravel the European coniferous forest refugia. The existence of European coniferous forest refugia is also supported by the East–West subdivision of several boreal species. Temperate refugia in Europe during cold periods might not have been restricted to the three Southern peninsulas. These refugia were most likely to be located near the Alps or in the Carpathians and, possibly, at the network of streams in the marginal areas of the Carpathian Basin.

The level of endemism generally correlates with the geological age of the refugia where relict-like taxa have been evolved and/or could survive. The Carpathian Basin belongs to the geologically youngest areas of Europe. Its relief developed under the influence of the Alpine orogenesis and by retreat of the Paratethys and the Pannonian inland sea. There are, however, some taxonomical groups which show considerable proportion of endemic species (land gastropods, earthworms or some soil arthropods). Most endemic species are narrow specialists inhabiting extreme habitats, e.g. thermal springs, karstic caves and karstic springs. A bulk of these endemic taxa is confined to the Eastern and Southern Carpathians, to the Apuseni Mts. and to the mountains of Banat, which could preserve relict species or some narrow endemics in refugia without permafrost phenomena during the last glaciations. Since the Carpathian Basin occupied a transitional position between the Balkanic refugia and the cold-continental tundra-steppe zone during the glacial periods, the post-glacial re-population of the Carpathian Basin was preceded (1) by long-distance dispersal from the more remote (atlanto- and ponto-) Mediterranean and Southern continental refugia, and (2) also from some adjacent local survival areas, e.g. from North-Western Balkanic (“Illyrian”) versus South Transylvanian (“Dacian”) arboreal refugia. In such cases, the arrows of the Northwards dispersal of the South-Western and South-Eastern populations surround the arid central part of the basin.

In the Carpathian Basin, the concentric arrangement of vegetation belts is influenced by numerous climatic, orographic, hydrographic and edaphic factors. The geographically transitional position of the Carpathian Basin resulted in a conspicuous mixture of faunal elements of diverse origins and geographical histories. The compartment structure of the vegetation complexes, typical of the Pannonian forest-steppe, has promoted the survival of very different faunal elements. Thus,

many thermophilic elements probably populated the Carpathian Basin not only by long-distance colonisation from remote, large glacial refuges, but also from numerous meso- or microclimatically favourable sites lying at the fluctuating borderlines of the Mediterranean refugial and periglacial belts. Eremic species are restricted to semi-desert-like habitats of the lowland with extreme edaphic conditions. Abundant examples can be found in strictly localised phytophagous insects, which are often connected with special halophytic plant communities. They are often represented by endemic Pannonian subspecies or allopatric sibling species of Turanian origin. The dispersal of this species group could have taken place in the late glacial phases on the Pannonian lowland with a subsequent isolation as a result of the post-glacial expansion of the forested belts. Two main groups of *xeromontane* elements are present in the Carpathian Basin. A larger number of species of the Mediterranean-xeromontane species belongs to some insect groups, e.g. Noctuidae and Orthoptera. The continental-xeromontane type is represented by some members of widely distributed Asiatic mountain steppe species and by some relict-like inhabitants of the rocky dolomit grasslands. It seems to be very probable that numerous genera, typical of the steppe biome, might have a xeromontane origin.

**Acknowledgements** I am deeply indebted to the precursors of modern phylogeographic thoughts: to the late Gustaf de Lattin and Willy Reinig who inseminated the biogeography by genetic insights and shaped my ideas. The Alexander von Humboldt Foundation repeatedly supported my research fellowships in Germany. The survey of the faunal history of Hungary was partly supported by the grant NKFP-3 B/023/2004.

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# Review

## The Dark Side of Relict Species Biology: Cave Animals as Ancient Lineages

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**Abstract** Due to their fascinating biology and phenomena belonging to the realm of scientific curiosity, cave animals have been objects of study for zoologists for numerous decades. This chapter not only focuses on the extremes (e.g., absence of eyes, specialization to extreme environments), but also serves as an introduction to understand the geographic distribution patterns and history of these highly diverse ecological groups with their relict characteristics. After an introduction to the subterranean environment in Sect. 1, we briefly review the biology and ecology of cave animals with their regressive and progressive evolutionary tendencies in order to understand the innate reasons for restricted distribution patterns (Sect. 2). In Sect. 3, we summarize the main aspects of our knowledge regarding the distribution of these species, especially in the Holarctic; and finally in Sect. 4, we highlight the relict characteristics of cave animal distribution and the ancient phylogenetic splits between cave and surface lineages.

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# 1 The Subterranean Environment

The environmental conditions of caves and other cavities in massifs have frequently been investigated in conjunction with studies on cave animals. The first studies were carried out in the 19th century in a region known as Karst or Kars (in Slovenia), located between Ljubljana and Trieste. It was rapidly understood that the so-called karst processes tend to create specific geomorphology and landscape with crevices, caves, dolines, or karrens when the land is formed by soluble rocks. In the case of the Slovenian Karst, the land is formed by carbonated rocks (mainly, limestone and dolomite), the type of rock which is most important worldwide for the development of caves. Other soluble rocks, such as evaporate rocks (e.g., gypsum, salty rocks, some quartzites), can also undergo karst processes, sometimes, however, only slowly. Today, a landscape in which the main features of rock, soil, and hydrology are predominantly caused by dissolution of bedrock, is called “karst”, from the Slovenian word for the above-mentioned region (Ginés and Ginés 1992).

Rock solubility is one of the main prerequisites for the formation of crevices and cavities. In the case of limestone, the main constituent is calcite which is not very soluble in pure water. However, under natural conditions water contains some acids, and this increases the solubility of calcite. The most important of these acids is carbonic acid, formed from water and carbon dioxide present in the atmosphere and soil (as a consequence of biological activity of roots, animals, protozoa, and bacteria). Other acids from the soil or from other rocks (e.g., humic acids) also have the potential to solubilize calcite. The dissolution of calcite and thus of limestone depends on several chemical dissociation and hydration reactions, both involving complex interplay of physico-chemical equilibria (Herman 2005) which, under specific conditions, cause secondary deposits in caves, e.g., stalagmites, flowstones, helictites, gypsum flowers, and columns.

The progressive dissolution of rock results in the development of fissures allowing infiltration of water into the inner parts of the karst massif, a process which tends to disarticulate the superficial hydrological network. This feeds the karst aquifer, and a subterranean hydrological network develops which promotes progressive dissolution and erosion of the inner parts of the karst massifs. Together with breakdown processes, rock solubility is the main force involved in the formation of characteristic karst phenomena such as caves, karrens, dolines, crevices, fissures, and karst springs (Culver and White 2005).

Caves can also be developed by other geological processes (e.g., pseudokarst, cryptokarst, or volcanism), but most caves are located on limestone in karstic areas. Since most work on cave animals has been conducted in limestone caves in temperate and Mediterranean climates, the following section also focuses on this type of habitat.

In general, the physical environment in caves varies much less than the surrounding and/or superficial habitats (Culver 1982; Culver et al. 1995; Wilkens et al. 2000; Culver and White 2005). The lack (or at least reduction) of diurnal and annual rhythms results in an environment without (or with strongly reduced) temporal coordination of animal activity. Especially, in the inner part of large caves or karst

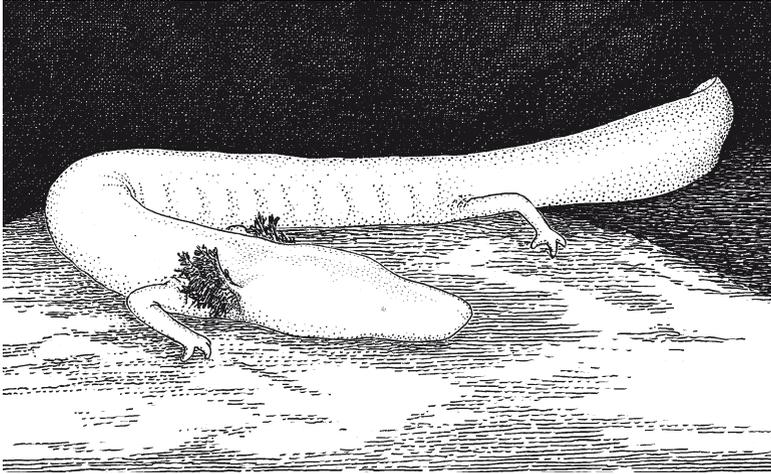
massifs the temperature varies less than 1°C in the course of the year and is, moreover, close to annual mean temperature (e.g., Juberthie 1969). Relative humidity and physico-chemical parameters of cave water are also relatively stable. Additionally, due to the decelerating effect of fissures and crevices, drainage basins in karst massifs have moderate flood peaks compared to other drainage basins (Culver 1982).

In the 1980s, the so-called superficial underground compartment (=“milieu souterrain superficiel,” MSS) was discovered in the Pyrenees and subsequently in many other regions with a temperate or Mediterranean climate. The MSS corresponds to both the underground compartment (caves) and the deeper horizons of the top soil. It consists of a system of heterogenous macroporal voids in rocky material underneath the soil (mesocavernous habitats; for a detailed description of the MSS, see Juberthie et al. 1980). Due to its intercrossing nature, between the underground and the surface, the MSS shows numerous intermediate conditions; however, the microclimate in some MSS seems to be more stable than in some large caves, due to the absence of air circulations which change in caves from winter to summer.

Due to the general absence of photoautotrophic organisms, cave communities and their food web are based on decomposers. Food enters caves only as organic matter in streams and vertical shafts (also via the MSS, see Gers 1998, as dissolved organic matter in water), and by feces of animals which regularly visit caves (e.g., bats). Only a few chemoautotrophically-based cave ecosystems have primary producers (e.g., Sarbu et al. 1996). In the underground and the superficial underground compartment, Collembola and Acarina function as “underground plankton” because of their basal position in many subterranean food webs. In general, decomposers (e.g., Diplopoda and Diplura) and first- and second-order predators (e.g., Coleoptera, Chilopoda, and Aranea) occur. For many cave animals, food is scarce and its availability is more stochastic and unpredictable in superficial habitats; its pattern of occurrence also seems to be more clustered (Gers 1998).

## 2 Biology and Ecology of Cave Animals

Cave animals are adapted to the above-mentioned special habitat conditions, which include reduced environmental variation and darkness. Loss of eyes, loss of wings in insects, and reduction of pigments are elements of regressive evolution; prolongation of extremities and increased sensory systems are elements of progressive evolution which occur in cave animals. The development of such features associated with cave life is known as troglomorphy (Christiansen 2005). In many cases, these features evolved convergently (e.g., the head flattening in cave vertebrates, a strong enlargement of elytra, but not of the abdomen – known as pseudophysogastry – in different lineages of beetles) and resulted in a special habitus of many cave-adapted animals (Figs. 1–3). These adaptations to the special environment are the reason why true cave animals are not able to survive outside of their underground habitats. The members of this ecologically characterized group which live exclusively in caves (or the MSS) during their whole life cycle are termed *troglobionts* or troglobites.



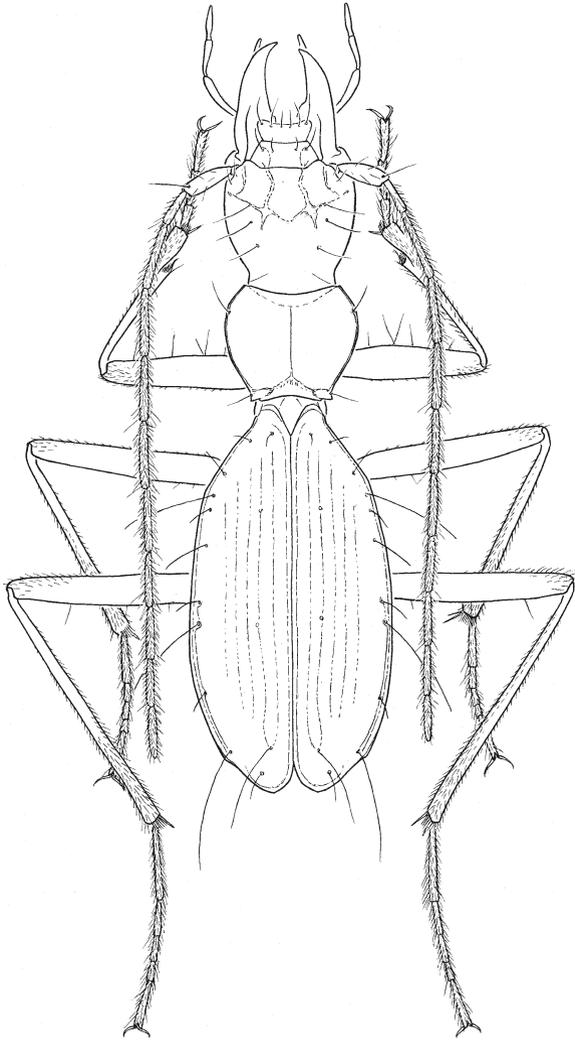
**Fig. 1** European cave salamander or olm (*Proteus anguinus*), larva (drawn by Peter Schüle)

*Troglophiles* are capable of spending their entire life cycles either in underground or in surface habitats. *Trogloxenes* are vagrants or animals which can spend part of their life in caves, but are not able to develop in the underground. The latter terms are mainly used for terrestrial species. *Stygobionts* or stygobites live exclusively in subterranean waters (including cave and interstitial habitats), whereas stygophiles are able to develop in subterranean and in surface water bodies (cf. Culver and White 2005).

Regressive evolution, in particular, is not yet well understood. The simple neutral mutation hypothesis implies that regression is caused by random mutations in genes involved in the development of a given character. Under the relaxed selective pressure of the cave environment, these random mutations can accumulate and lead to the loss of the given character. Jeffery (2005, 2008) reviewed studies of regressive evolution in the Mexican Blind Cavefish (*Astyanax mexicanus*) and concluded that pigmentation regression is probably best explained by the neutral mutation hypothesis, whereas eye degeneration may be caused by adaptive evolution and pleiotropic effects of the genes also involved in the development of eyes. A review on the regressive evolution of cave fishes, in general, is given by Jeffery (2009).

As a consequence of eye reduction, the circadian rhythm of many cave animals is also reduced. Extremely evolved cave animals exhibit no or only weakly developed circadian rhythms of locomotory activity and resting. However, it was shown that light can induce clear activity rhythms in troglobite species (Lamprecht and Weber 1979, 1992).

Phenomena of progressive evolution characterize many cave animals. The prolongation of extremities in arthropods and the increased number of receptors for environmental information (especially, for olfactory stimuli) are features of most cave animals (Juberthie 1979; Culver 1982; Christiansen 2005). The spectrum of



**Fig. 2** Carabid cave beetle *Aphaenopidius kannikensis*. Adapted from Assmann and Lompe (2006) and Drovenik et al. (2008), modified

consumed food ranges from feces, litter, and rotten wood to living prey. Some predators are specialized (e.g., *Neaphaenops tellkampfi* with its preference for eggs of the cave cricket *Hadenoeceus subterraneus*, Kane and Poulson 1976), while some others are polyphagous (e.g., *Aphaenops crypticola*, Gers 1996). The reduced and stochastic availability of food seems to have resulted in selection pressure to a lower metabolic rate which allows both greater resistance to starvation and a comparably higher availability of energy for reproduction. Generally, but not in all

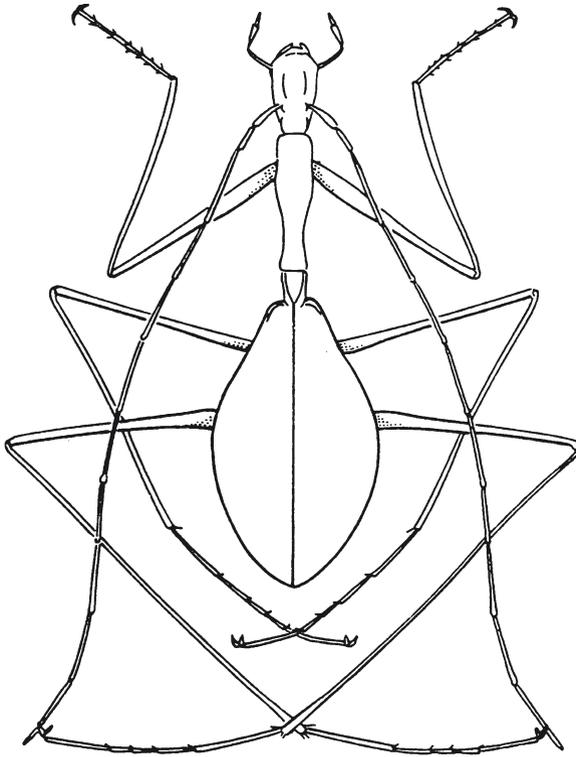
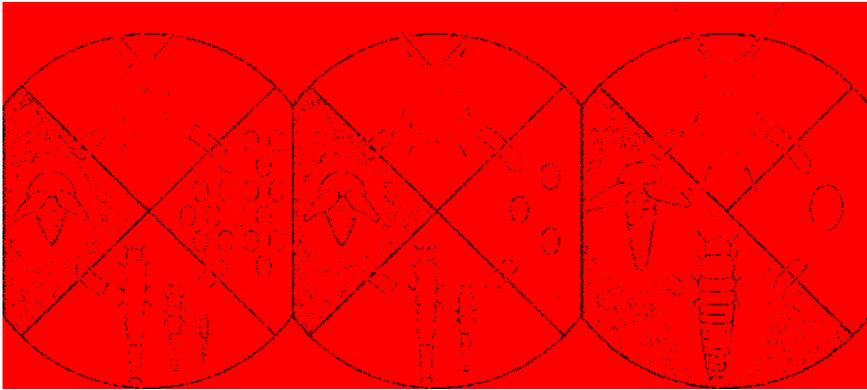


Fig. 3 Cholevid cave beetles *Antroherpon dombrowski*. Adapted from Jeannel (1924)

animal groups, cave species show a lower metabolic rate than their relatives that inhabit epigeic habitats (Vandel 1965; Culver 1982; Hüppop 2005).

The life span of many cave animals seems to be prolonged: The olm or European cave salamander (*Proteus anguinus*) can become older than a century (Durand 1998), and mark-recapture experiments with the microphthalmic cave ground beetle *Laemostenus schreibersi* in an Austrian cave estimated an age of more than 8 years for some individuals (Rusdea 1994, 2000). Moreover, cave animals have the tendency to produce fewer, but larger eggs and/or offsprings (Hüppop 2005). K-selection (cf. longevity, reproduction biology) seems to act on animals in the underground (see Stearns 1977 for a critique on the concept of r- and K-selection). Some members of the cholevid beetle genus *Speonomus* from the Pyrenees are an extreme example for the tendency to invest more yolk in fewer eggs. In the most extremely adapted species, only one egg ripens in a female at any given time. The hatched larva does not feed, grow, or molt, but pupates rapidly. Thus, the cycle of these species comprises only a single larval stage. This means that female investment in one egg enables the development of a whole beetle (Fig. 4). Transitions to the classical life cycle of cholevid beetles with three successive larval stages are also known, however (Glacon-Deleurance 1963).



**Fig. 4** Life cycles of different Cholevidae, Leptodirinae. Adapted from Glacon-Deleurance (1963) and Giachino et al. (1998), redrawn by Peter Schüle

### 3 Biogeography and Diversity Hotspots

The marked differences between the distribution patterns of aquatic and terrestrial troglobites were recognized by biologists several decades ago (Thienemann 1950; Vandel 1965; Lamoreux 2004). The Northern distribution limit of terrestrial troglobites does not reach as far North as that of aquatic troglobites, at least in Europe and North America. For example, members of *Niphargus*, an aquatic amphipod genus with blind species which inhabit groundwater in caves and other geological settings, are distributed Northwards as far as Ireland. Terrestrial forms (e.g., some genera and many species of blind cave Trechini beetles), however, reach their Northern limits in the so-called “massifs de refuge” at the Southern border of the Alps, in small and isolated refugia at the North-Western and North-Eastern border of the Alps, and in the Carpathians (Holdhaus 1954; Vandel 1965). The reasons for the different distribution patterns are still under discussion (cf. Lamoreux 2004).

The Southern edge of permafrost ground coincides notably with the Northern edge of the distribution range of terrestrial troglobites (Holdhaus line; see for a detailed discussion Drees et al. 2009). Due to the narrow adaptations to their habitats, terrestrial cave animals are not able to leave the massifs in which they live. Power of dispersal is, therefore, extremely restricted and it is highly probable that terrestrial cave animals survived the glacial periods within the massifs in which they are still distributed. Exceptions are known where the species have been able to colonize from nearby caves or massifs, probably using the MSS (cf. Daffner 1993).

Cave animals belong to the trophic groups of decomposers and carnivores, and their food chain depends on organic input from epigeic parts of the ecosystem. Survival of terrestrial cave animals during the glacial periods indicates a long-lasting persistence of ecosystems in the given regions.

The postglacial colonization of Eastern North America seems to be very complex, and many questions remain unanswered. However, it is known that glacial refuges

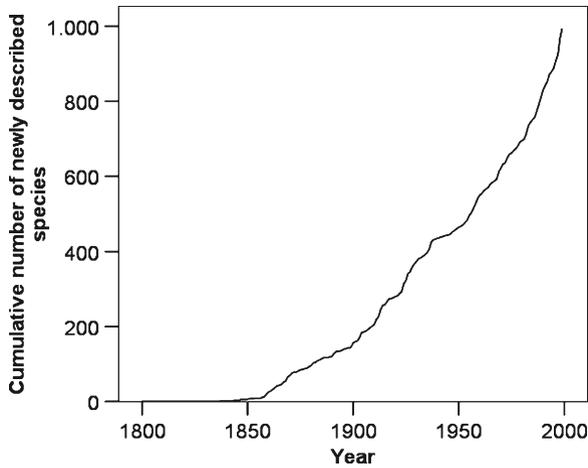
were localized in areas Northwards up to close to the Laurentide Ice Sheet (Soltis et al. 2006), and terrestrial cave animals were restricted to non-glaciated areas, with a concentration in Alabama (Culver et al. 2000). Some regions of the world lack a terrestrial cave fauna (e.g., Northern Asia), while in others their existence has been discovered only in recent decades (e.g., in the tropics, Howarth 1983). A general overview of the worldwide distribution of terrestrial and aquatic cave animals is given by Juberthie and Decu (1998).

Bizarre disjunct distribution patterns in North America and in Europe can be found in higher amphibian taxa with troglobitic or at least troglphilic species: The family of cave salamanders (Proteidae) comprises the genus *Necturus* in surface waterbodies of North America and the European cave salamander or olm (*P. anguinus*) Southeast of the Alps in Europe; troglphilic lungless taxa inhabit disjunct distribution areas in Western North America (*Hydromantes*) and in the North-Western Mediterranean (*Speleomantes* and *Atylodes*; Durand 1998; Weisrock et al. 2005). Disjunct distributions are also known from groups of blind ground beetles which occur in North America and in the Palearctic (the so-called “série phylétique” of *Aphaenops*, Trechini; Casale and Laneyrie, 1982). Such disjunct small distribution areas on two continents are strong arguments for ancient splits in the phylogeny of the given groups. Without doubt, these taxa are ancient relicts of a former wider distribution.

One of the “hottest hotspots” of terrestrial cave fauna is located in the Western Palearctic realm, probably with a center in the Northern part of the Mediterranean region (Culver and Sket 2000; Gibert and Culver 2005; Culver et al. 2006). Ground beetles are a good example of “clustered” cave species richness: Casale et al. (1998) cite more than 50 genera of troglobitic ground beetles of the tribe Trechini from the Mediterranean realm (mainly from Southern Europe; and 13, 4, and 7 genera from North America, South America, and Australia, respectively). Another important hotspot seems to be Southeast Asia (more than 30 genera listed by Casale et al. 1998, and numerous newly described taxa, cf. Lorenz 2005). More than 600 of a total of more than 1,000 troglobitic trechine species from the Western Palearctic have been described.

The species number of some cave genera is low and there are even some monotypic genera (e.g., *Amerodualius*), whereas others are extremely rich in species, e.g., troglobitic ground beetles with >250 *Pseudanophthalmus* species in North America, and a similar number of *Duvalius* species in the Palearctic (Casale et al. 1998; Culver et al. 2003). Extraordinary species richness is also a feature of some aquatic cave animal groups, especially of the amphipod families Niphargidae and Crangonyctidae (both *Niphargus* and *Stygobromus* comprising about 200 species in Europe and North America, respectively, Peck 1998; Gibert and Culver 2005). In general, however, the species richness of aquatic fauna is lower than that of terrestrial species (Culver et al. 2003). The reasons for the different species richness patterns have not yet been conclusively ascertained (cf. Lamoreux 2004).

The invertebrates inhabiting the underground are still poorly studied, from a systematic point of view. Species richness of ground beetles inhabiting underground habitats in the Palearctic has not yet reached an asymptote (Fig. 5), whereas overall species richness of carabids in most European and North African countries reached a clear asymptote during the 20th century (Schuldt and Assmann 2009).



**Fig. 5** Cumulative number of carabid taxa inhabiting underground habitats (troglobionts and troglaphiles, including blind species from the superficial underground compartment) described from the Palearctic (1800–1999). Generated from the Catalog of Palearctic Coleoptera, Löbl and Smetana (2003)

#### 4 Relict Status of Cave Animals

Biospeleologists agree that cave animals share a common ancestor with epigeic species. These ancestors lack features of progressive and regressive evolution which is related to the progress of troglobite evolution. According to the “Pleistocene-effect theory”, ancestral forms are thought to have adapted (“taken refuge”) to caves when “postglacial climates became warmer and drier, and thus inimical to ancestors preadapted to cool, wet microhabitats” (Vandel 1965; Culver 1982; Barr and Holsinger 1985 p. 331; Moldovan 2005).

This scenario does not seem to be true, however, from modern molecular studies on many cave animals. The DNA divergence of studied taxa indicates that biogeographic events which led to the separation of the gene pools date back some millions of years (Caccone and Sbordoni 2001; Porter 2007). Even surface and cave *Astyanax* populations seem to have diverged at least a few million years ago (Strecker et al. 2003; Strecker et al. 2004; 1.8–4.5 million years: Porter et al. 2007).

At least one population of the isopod *Asellus aquaticus* is an up to 3.9 million years old PrePleistocene “relict” of one lineage which invaded the underground. Other cave populations diverged during the glacial periods (Verovnik et al. 2004). However, despite the fact that we have molecular data only from a few related cave and surface populations/species we have to assume that the origin of many terrestrial and aquatic cave animals dates back to times (long) before the glacial periods.

Thanks to molecular studies, we are beginning to understand the evolution of speciation and colonization processes (e.g., Verovnik et al. 2005), but our knowledge of these processes in terrestrial species is still poor. Erwin (1985) developed

the taxon pulse model to explain the divergence of the hyper-diverse group of ground beetles and Casale et al. (1998) adopted it to stress the origin of subterranean lineages. However, despite its sophisticated and outstanding character, a molecular test using arboreal and epigeic ground beetles did not support the taxon pulse model (Ober 2003).

Cave animals are treated as relict species for good reasons, because in many cases they are taxa with no ancestral stock in the immediate area (Barr 1985). Taking the long-lasting divergence since the PrePleistocene times between many cave and surface lineages into account, we can denominate many ancient relicts among cave animal species. This also seems to be true for taxa with striking disjunct distributions (see biogeography and biodiversity hotspots, Sect. 3): The split from the common ancestor of *Hydromantes* (North America) on the one hand and *Speleomantes* and *Atylodes* (Europe) on the other hand occurred during the Paleocene-Eocene and both lineages can be named relicts, not only because of their ancient origin but also because of their restricted distribution areas (Vieites et al. 2007).

Despite our understanding of the biogeographic history of cave animals, numerous questions are still unanswered. Some have already been mentioned above, such as the reasons for different distribution and species richness patterns between terrestrial and aquatic troglobionts or the reason for the existence of regions without cave fauna (South of permafrost grounds during glacial periods). In addition to these gaps in knowledge, the phylogeny of many vertebrate, and even more invertebrate, species remains to be studied. For example, phylogeny is still unresolved for the European cave salamander or olm (*P. anguinus*) (Weisrock et al. 2005) described as the first eyeless cave animal by Laurenti in 1768, even though this bizarre species was already studied by Cuvier, Lamarck, and even Darwin in his famous book on “The origin of species,” already one and a half centuries ago.

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# Relicts Within the Genus Complex *Astragalus/Oxytropis* (Fabaceae), and the Comparison of Diversity by Objective Means

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**Abstract** *Astragalus* and *Oxytropis* represent one of the largest angiosperm genus complexes. It is widely discussed why – and if at all – it contains so many species. Here, we discuss how the genetic divergence in a postglacial relict species, *Oxytropis pilosa*, can provide insights into the relative genetic variance of taxa. Methods are introduced to obtain sequence clusters of similar genetic diversity and to detect statistically significant differences. These approaches provide objective means to identify taxa that deserve the same protection efforts, independent of a fixed species concept. *O. pilosa* ITS sequences display an inter- and intra individual variance, comparable to clusters comprising numerous accepted species. Regarding genetic diversity, some *O. pilosa* populations represent relicts that deserve the same or a higher protection status than some species of *Astragalus*. We discuss the need of objectivity in identifying the evolutionary hot spots as well as the measures to define and protect them.

## 1 Introduction

*Astragalus* (~2.500 species; Podlech 1986) and *Oxytropis* (~300 species) are closely related genera, representing one of the largest angiosperm genus complexes. Taxonomic instability within the genus complex comprising both *Astragalus* and

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*Oxytropis* results in a high number of similarities, which may differ in their assignment to the genus. In fact, the separation of both genera is based on a single morphological criterion only: *Astragalus* exhibits a mucronate keel along the adaxial side of the legume, whereas *Oxytropis* is characterized by an abaxial carinate legume (Candolle 1802; Barneby 1964; Chater 1968). Molecular studies encountered difficulties in providing sufficient resolution at the species level, independent of the sequence marker used (nuclear and/or chloroplast DNA spacer: Sanderson 1991; Sanderson and Doyle 1993; Wojciechowski et al. 1993, 1999; Kazempour Osaloo et al. 2003). Efforts in chemotaxonomy were promising (Williams and Barneby 1977), but have been only sporadically applied (Tin et al. 2007).

It has been widely discussed why so many species are recognized for *Astragalus* (Sanderson and Wojciechowski 1996), but it has seldom been addressed whether this large number of species, which is continuing to increase in local floras, optimally represent the natural relationships. *Astragalus* underwent a considerable radiation in all its main centers of biodiversity: Eurasia (mainly Southwest Asia), Africa, North America, and South America. The high number of morphologically defined species – and varieties – is caused by numerous endemics, often referred to as “relict” species because of their isolated mountainous stands and small populations. It is rare to find widespread *Astragalus* species. *Astragalus* covers various climates within its biogeographical areas, such as continental to arctic steppes, stony alpine slopes, and Mediterranean mountainous rocky places or even tropical African highland (Barneby 1964; Gillett 1964; Chater 1968). The preference for mountainous habitats, in conjunction with (post)glacial rapid environmental changes, may have facilitated differentiation processes leading to a split up into numerous species and varieties.

*Oxytropis* shows a quite similar distribution area in Eurasia and North America compared to *Astragalus*, but is absent from South America. This distribution is comparable to that of another large genus – *Pedicularis* (Sauer et al. 1990). *Oxytropis* exceeds more to the (arctic) North of the Northern Hemisphere than *Astragalus*, and some remarkable species such as *O. campestris* with its distinct sub species can be found on both continents. *Oxytropis pilosa* (Fig. 1) is a rare relict species exhibiting a highly fragmented distribution area. It is mainly found in the Pannonian Basin (Hungary to Austria) and in the Alps with prompt occurrences in Scandinavia (including the seashore of Gotland), the Baltic as well as in the Abruzzo (Hegi 1924; Meusel et al. 1965; M. Schlee, pers. observation). In a number of other accounts including *O. pilosa* (M. Schlee, G.W. Grimm, M. Göker, V. Hemleben, in prep.), the internal transcribed spacers (ITS1 and ITS2) of the nuclear ribosomal DNA (nrDNA) allowed reconstruction of detailed evolutionary pathways (Schlee et al. 2003; Denk and Grimm 2005; Grimm et al. 2007) as they are biparentally inherited, part of a multicopy gene region and undergo (to some degree incomplete) concerted evolution as well as frequent intragenomic recombination (Arnheim et al. 1980; Hemleben et al. 1988; Baldwin et al. 1995; Volkov et al. 2007). We detected significant intraspecific and intraindividual genetic variability in *O. pilosa* by cloning and sequencing a large number of nrDNA internal transcribed spacer sequences (ITS1 and ITS2; M. Schlee, G.W. Grimm, M. Göker,



**Fig. 1** *Oxytropis pilosa*, xerothermic habitat on gypsum, Ascoli Piceno, Marche, Italy

V. Hemleben, in prep.). The variability found allows discussion of migratory and evolutionary patterns for this species, which will form the subject of an upcoming study.

Here, we make use of the large amount of sequence data obtained for *O. pilosa* to conduct a comparison of the genetic diversity of clades by objective means. This is done to find an alternative to classical methods, such as morphotaxonomy-based approaches for the identification of units that represent hot spots of biodiversity (here, genetic resources). We apply non-hierarchical single-linkage clustering to the combined *Astragalus* and *Oxytropis* ITS dataset after determining the distance threshold that optimally delimits *O. pilosa*. Thus, the resulting clusters represent estimates for species boundaries obtained by the use of an operational and fully-automated method treating *O. pilosa* as the standard. The clusters are expected to be uniform, given the distance threshold and the clustering algorithm (i.e., non-hierarchical single-linkage, NHSL). The obtained clusters are discussed with respect to the taxonomic affiliation (assigned species) of the comprised sequences. A character-based approach is used to determine whether the genetic diversity of selected clusters is significantly distinct. If one agrees that in conservation management major emphasis needs to be laid on equal protection efforts for taxa that are equally diverse (Watt 1947; Stock et al. 1999; Potthast 2000; Schlee 2004), it is crucial to compare diversity by appropriate means. Taking into account that taxonomic affiliations and taxonomic ranks often only poorly reflect relative genetic variance, we wonder whether they are sufficient to determine valuable genetic resources. Here, we thus address the question if biodiversity assessment based on substantial molecular data is superior to estimating diversity from data linked to the recognition of morphospecies and if the proposed techniques are valuable to estimate the relative need for conservation efforts as a means to discriminate protection units. Finally, we

briefly discuss the conservation measures that are appropriate in the case of *O. pilosa*, once the most valuable genetic resources have been identified.

## 2 Material and Methods

In addition to our own dataset of *Oxytropis* ITS clones comprising the ITS1, 5.8S rDNA, and ITS2 regions, and flanking 18S and 25S rDNA (also including some European *Astragalus*; EMBL accession numbers AM401376 to AM401574; AM943374 to AM943384; FM205750 to FM205773), we added all ITS sequences stored in DDBJ, EMBL, and NCBI gene banks (sequences usually obtained by direct sequencing, not by cloning; downloaded on January 2008) for the genera *Astragalus* and *Oxytropis*. Solitary ITS1 and ITS2 sequences were combined if they belonged to the same source according to the original literature/description. The sequences were aligned using the POA program (Lee et al. 2002). The 5.8S rDNA was excluded from the analyses. Phylogenetic inference was performed under the maximum likelihood (ML) optimality criterion using RAxML 7.0 (Stamatakis 2006; Stamatakis et al. 2008). The program implements a new fast ML bootstrapping and subsequent search for the best topology. Duplicate sequences were eliminated prior to ML analyses. Tree inference and 100 bootstrap replicates were conducted under the CAT approximation (Stamatakis 2006), but final parameter optimization was done under a GTR+ $\Gamma$  model. A plain text (NEXUS) file containing the complete alignment, GenBank accession numbers, and information on the sets of identical sequences reduced by RAxML to a single one, respectively, is available at <http://www.goeker.org/mg/clustering/>.

To obtain a non-nested sequence classification based on the given distance (or similarity) threshold, we use NHSL clustering, as in the case of the popular tool blastclust (<ftp://ftp.ncbi.nih.gov/blast/documents/blastclust.html>; regarding hierarchical single-linkage clustering, see, e.g., Legendre and Legendre 1998, pp. 308–312). NHSL is based on the notion of a “link”. A link is defined as any distance between two objects (here, sequences) that is smaller than or equal to the predefined threshold. NHSL starts by assigning the first object to the first cluster. For each of following objects in turn, it assigns that object to the same cluster than a previously clustered object if the distance between them is a link. If no such previously classified object is found, the current object is assigned to a new cluster. If several such previously classified objects are found that belong to distinct clusters, these clusters are joined.

Obviously, larger threshold values will result in larger but less numerous clusters, whereas small thresholds will lead to numerous small clusters. A biologically sensible threshold is usually not known a priori. However, a particularly well studied (and monophyletic) taxon (here, *O. pilosa*), can be used as a standard by determining the lowest possible threshold that results in all sequences obtained from that taxon being assigned to a single cluster. It is easy to implement an algorithm that calculates this value for a predefined group. Wirth et al. (1966, p. 61) who used similarities

instead of distances define "...a similarity value,  $c$ , which is the largest fixed linking similarity value for which the cluster is still an interlinked aggregate of specimens". For each of the  $n$  objects within the group, the distance to the least distant object that belongs to the same group is determined; the largest of these  $n$  values represents the result, analogous to  $c$ . This is due to the fact that in NHSL, a *single* link is sufficient for an object to be assigned to a cluster. This algorithm and NHSL have been implemented in the program OPTSIL (Göker et al. 2009) which is available upon request. The determination of the standard threshold and NHSL relied on uncorrected distances (also called "Hamming" or " $p$ " distances; e.g., Swofford et al. 1996, p. 455), which represent the relative number of deviations between two sequences. For downloaded sequences, we relied on the NCBI taxonomy provided in the same files to assign them to genera and species. The optimal threshold calculated for *O. pilosa* was then applied in NHSL.

A disadvantage of the NHSL approach is that clustering methods such as single-linkage clustering cannot be considered as valid methods of phylogenetic inference, mainly because lower pair wise distances (or higher similarities) do not necessarily indicate a closer phylogenetic relationship (e.g., Felsenstein 2004, pp. 165–167). This issue has led to the widespread avoidance of UPGMA clustering (Sokal and Michener 1958) in phylogenetic studies. However, the difficulties may be tempered or even disappear if non-nested clustering is applied and if species are to be distinguished, because within-species sequence dissimilarities are expected to be rather low. The same rationale applies to the usual arguments against uncorrected distances because unobserved, superimposed nucleotide substitutions only play a role if distances are large (e.g., Felsenstein 2004, p. 158).

Göker and Grimm (2008) used the well-known Shannon entropy formula (Shannon 1948) to calculate the character data of *hosts* (plant individuals) from the character data of their *associates* (cloned sequences obtained from the respective plant individuals). For all sequences belonging to the same individual, the entropy of each alignment column was calculated to represent the amount of genetic divergence within each individual using the program G2CEF designed and implemented by M. Göker (downloadable from <http://www.goeker.org/mg/distance/>). An alignment of length  $n$  will thus result in  $n$  corrected entropy values per group. Because the variance in nucleotide characters may depend on the number of sequences, each entropy value was corrected by division through the maximum possible entropy for the given number of associates, which is 0 in the case of a single associate. Accordingly, the corrected entropy values for individuals present with a single sequence only are undefined.

For each pair of groups,  $n$  differences between the  $n$  corresponding corrected entropy values can be determined. Subsequently, a non-parametric Wilcoxon signed-rank test or a parametric t-test (or any other appropriate statistical test) can be applied to assess whether the distribution of these differences significantly deviates from 0 and, thus, the two original entropy distributions are significantly different from each other. This procedure is similar to paired-site tests used to assess whether the scores of two phylogenetic trees are significantly different, given a sequence alignment of the same taxa (Felsenstein 2004, p. 364 ff.). Pair wise tests

were conducted with R (R Development Core Team 2005) and restricted to those clusters obtained by NHSL that comprised more than five sequences.

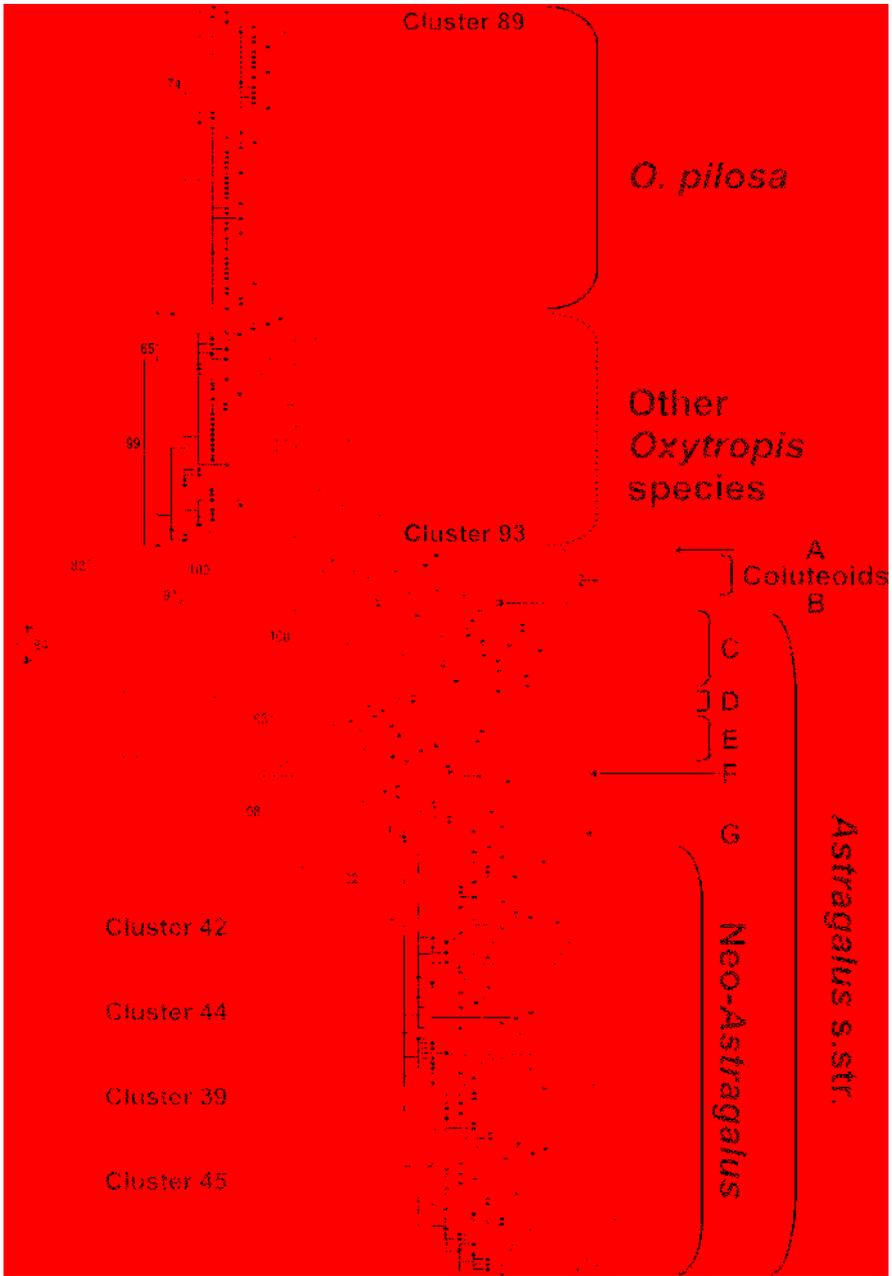
### 3 Results

Our final data matrix comprised 437 ITS sequences of *Astragalus* and *Oxytropis* species comprising 464 characters from ITS1 and ITS2. Of these 437 sequences, 184 were exact sequential duplicates of others; 291 of the 464 characters were variable and 195 were parsimony-informative. After elimination of duplicates, the alignment analyzed under ML comprised 358 distinct alignment patterns, including a proportion of gaps and completely undetermined characters of 7.48%. The best ML tree obtained is shown in Fig. 2. The tree was rooted along an unresolved backbone polytomy giving respect to a bipartition that separated all *Astragalus* s.str. from the remaining *Astragalus* lineages and *Oxytropis* (in 98% of ML bootstrap replicates). The remaining backbone could be considered to be rather well-resolved, indicating several lineages with bootstrap support (BS) between 70 and 100. Towards the tip of the tree, a general decrease in BS was observed (details not shown). Particularly, the well-known *Neo-Astragalus* clade received little BS. Multiple accessions of the same *Astragalus* species (also listed in Table 1) were found within the same clade (with varying support), as were all ITS sequences of *O. pilosa* (BS=74, new cloned data plus one directly sequenced NCBI GenBank accession). In contrast, the two other extensively sampled species of *Oxytropis*, *O. arctica* and *O. campestris*, were intermixed with each other and with several other *Oxytropis* species.

Optimal distance thresholds calculated for the present species are shown in Table 1. We obtained an optimal value for *O. pilosa* of about 0.0069. Results of NHSL obtained with the optimal threshold are shown together with the ML tree in Fig. 2.

The largest clusters and their properties are shown in Table 2. *Oxytropis campestris* appeared inseparable from *O. arctica* as well as from a number of less intensively sampled *Oxytropis* species. The clusters 39, 42, 44 and 45 each contained a number of distinct *Astragalus* species, indicating that genetic divergence within many accepted *Astragalus* species is much lower than within either *O. pilosa* or the *O. arctica*–*O. campestris* complex.

The results of the Wilcoxon signed-rank and t-tests, which are shown in Table 3, confirmed this conclusion. According to the Wilcoxon test, genetic divergence of *O. pilosa* was not significantly different from the *O. arctica*–*O. campestris* complex, but significantly different (lower) from *Astragalus* clusters 44 and 45 (species numbers given in Table 2), and significantly different (higher) from *Astragalus* cluster 42 and *Astragalus* cluster 39, respectively. According to the t-test, genetic divergence of *O. pilosa* was not significantly different from any of the other clusters, whereas the *O. arctica*–*O. campestris* complex was significantly more divergent than *Astragalus* clusters 42 and 39, respectively.



**Fig. 2** ML phylogram based on ITS data of *Astragalus* and *Oxytropis*, with major clusters (see text) and clades annotated. Numbers at nodes indicate non-parametric bootstrap support based on 100 ML bootstrap replicates. Clusters (see text) are highlighted by distinct background shading, clade names are given at right. A, *A. vogelii*; B, *A. epiglottis* and *A. pelecinus*; C, clade of Eurasian *Astragalus*; D, *A. corrugatus* and allies; E, clade of Eurasian and North American *Astragalus*; F, *A. alpinus*; G, *A. adsurgens* (cf. Table 1)

**Table 1** Optimal distance thresholds (i.e., smallest thresholds for which the group is retained in a non-hierarchical single-linkage clustering; NHSL) for the *Astragalus* and *Oxytropis* taxa present in the molecular dataset

Taxon	Optimal threshold	Number of sequences
<i>A. hoantchy</i> , clade C	0.01609195	3
<i>A. didymocarpus</i> , <i>Neo-Astragalus</i> clade	0.01395349	4
<i>A. adsurgens</i> , G	0.01173709	2
<i>O. campestris</i>	0.01162791	23
<i>A. lentiginosus</i> , <i>Neo-Astragalus</i> clade	0.00697674	6
<i>O. arctica</i>	0.00692841	18
<i>O. pilosa</i>	0.00688073	200
<i>A. membranaceus</i> , clade C	0.00458716	3
<i>A. alpinus</i> , F	0.00440529	2
<i>A. layneae</i> , <i>Neo-Astragalus</i> clade	0.00232558	3
<i>O. halleri</i>	0.00229358	7
<i>A. complanatus</i> , Coluteoid clade	0.00229358	2
Others	0	1

An optimal threshold of 0 is due to the presence of no more than a single sequence for the respective taxon or due to all sequences being identical. Single uppercase letters (C, F, G) after the taxon name refer to Fig. 2

**Table 2** Clusters containing more than five sequences obtained by using non-hierarchical single-linkage clustering and the optimal distance threshold determined for *O. pilosa*

Cluster no.	Number of species	Number of sequences contained	Taxonomy	Average corrected entropy
89	1	200	<i>O. pilosa</i>	0.005865
93	16	59	<i>O. arctica</i> , <i>O. campestris</i> , <i>Oxytropis</i> spp.	0.009686
44	19	19	<i>A. spp.</i> ( <i>Neo-Astragalus</i> )	0.008344
45	14	16	<i>A. spp.</i> ( <i>Neo-Astragalus</i> )	0.008122
42	13	16	<i>A. spp.</i> ( <i>Neo-Astragalus</i> )	0.002533
39	12	16	<i>A. spp.</i> ( <i>Neo-Astragalus</i> )	0.002326

The reference numbers of each cluster, the numbers, and the taxonomic affiliations of the contained sequences, and the average corrected entropy values are shown

**Table 3** Results of the Wilcoxon signed-rank tests (lower left half) and t-tests (upper right half) applied to the five largest clusters found by NHSL

Cluster no.	89	93	44	45	42	39
89	–	0.1466	0.4906	0.4288	0.08392	0.2013
93	0.4882	–	0.7527	0.6992	0.0005*	0.0279*
44	0.0099*	0.7671	–	0.9497	0.0291*	0.0694
45	0.0025*	0.4939	0.7987	–	0.0326*	0.0853
42	<0.0001*	<0.0001*	0.1052	0.0393*	–	0.9373
39	<0.0001*	<0.0001*	0.004*	0.0160*	0.3964	–

Differences significantly ( $p \leq 0.05$ ) deviating from 0 are marked with an asterisk

## 4 Discussion and Conclusion

Genetic variability of populations within the *Astragalus-Oxytropis* complex has most often been addressed by techniques, such as allozyme polymorphisms (Karron et al. 1988; two endemic and two widespread species), RFLP (Kulshreshtha et al. 2004; one *Astragalus* species comprising six varieties, two *Oxytropis* species, pea as outgroup), AFLP, or RAPD (Travis et al. 1996; one variety). Only a few studies used cloned ITS sequences to compare populations (Dong et al. 2003, eight widespread species, one comprising two varieties, another comprising two sub species; Yip and Kwan 2006, one widespread species from 23 localities divided into at least two undefined varieties). Both studies found the marker highly conserved, and proposed the use of 5S rDNA spacers for the future. In contrast, our results indicate that if a sufficiently large number of cloned sequences are retrieved and if populations are sampled from a broad biogeographic range, valuable insights into the genetic divergence of populations can be obtained. Such substantially sampled taxa (here, *O. pilosa*) can be used as standards to optimize distance thresholds for non-hierarchical clustering algorithms. The adapted clustering techniques will result in a division of sequences into subsets of comparable genetic divergence as a basis to define conservation units. Statistical tests based on multiple sequence alignments allow us to compare the molecular diversity of different groups by objective means and to identify the significant differences regarding genetic variance. In contrast, traditional approaches such as AFLP, RAPD, RFLP, and microsatellite studies are often methodologically restricted to the species level and are statistically difficult to interpret (Felsenstein 2004, pp. 240f, 246f).

Results of our molecular comparison approach are in agreement with previous studies. For instance, the fact that *O. campestris* and *O. arctica* are hardly separable has been addressed by Jorgensen et al. (2003). Likewise, the considerably high genetic variance observed in our *O. pilosa* data confirms the interpretation of that species as a relict species. Particularly, the comparison of *O. pilosa* and the *Neo-Astragalus* species underlines how much divergence is preserved within the former. In fact, single populations of *O. pilosa* are as divergent as or even considerably more divergent than some *Astragalus* species. As a consequence, they deserve the same conservation efforts as the latter. This result is striking regarding the tendency in literature to suggest the protection of even single alleles within a variety of *A. cremnophylax* (Travis et al. 1996; for further examples, see the discussion in Soltis and Gitzendanner 1999), one of the numerous species included in our cluster 44 (Fig. 2 and Tables 1–3). Already the limited data available on *Astragalus* indicates that some species within the *Neo-Astragalus* clade (Table 1) are genetically more diverse than others, which should be taken into account in course of conservation.

In our view, not each unique individual and/or morphotype should per se be considered a relict or endemic. Biodiversity hotspots and genetic resources can be detected by objective means, similar to the approach presented here. Suitable nature conservation efforts should, therefore, also rely on molecular markers and algorithms that identify those relict stocks with the highest potential for ongoing speciation

processes, and those stocks that are endangered to become extinct. A highly adaptive species complex will frequently evolve (and lose) new, unique, and locally restricted morphotypes, but will not necessarily significantly increase its genetic diversity (Table 3). As a consequence, such a species complex will show a high biodiversity as reflected by the number of accepted (endemic) “species”, with only little genetic divergence between them. On the other hand, true relict species such as *O. pilosa* are morphologically and ecologically restricted or conservative (Hegi 1924), but can harbor a high genetic diversity (cf. Tables 1–3).

Focusing on the genetic diversity within the ITS regions may only be part of the story. Most likely, amplifying additional genes will result in additional insight into ecology and morphology. However, because of a lack of both time and funding, it is hardly realistic to do such multi gene sequencing for a broad sample size, and to detect and protect each individual that is a potential founder organism. Rather, one should focus on the identification and protection of at least some of the remaining true relicts in our rapidly changing world. Even though the cost of sequencing is continuously decreasing (because of recent advances in high-throughput techniques), there will always remain a trade-off between the number of loci amplified per individual and the number of individuals examined. As demonstrated above, it is easy to quantitatively determine units with similar genetic diversity, as long as a sufficient number of sequences is amplified for an appropriate reference taxon (here, *O. pilosa*). Accordingly, focusing on a single molecular locus, preferably one for which a large number of sequences are already deposited in public databases, is likely to remain the method of choice in many future studies. Once the most valuable genetic resources have been identified, using the methods described above (or any other appropriate means), protection measures for these resources can be laid out. Phytosociological studies can provide valuable insights into a species’ habitat needs. Such studies are necessary, particularly if the genetic divergence within single individuals is so high that they deserve considerable conservation efforts, as confirmed by the present study for *O. pilosa*. In such a case, conservation should aim at preserving as much of the estimated genetic richness as is possible. The monitoring of relicts should be intensified, in general, using the combination of molecular and phytosociological methods to detect the evolutionary hotspots and endangered genetic resources as well as the means to protect them. Phytosociological surveys were conducted for *O. pilosa* using the Braun-Blanquet approach to quickly obtain detail-rich relevées (Schlee et al. 2003; M. Schlee, G.W. Grimm, M. Göker, V. Hemleben, in prep.). As a conclusion from these surveys, conservation efforts need to include more suitable land-use of the habitats. A management system should prefer a grazing over a cutting regime. The toxicity of *Astragalus* and *Oxytropis* species, in general, is here of lesser importance because food aversions can be conditioned if they do not already exist in grazing animals (Ralphs and Provenza 1999). Hairiness of the plants (e.g., *O. pilosa*) or spines (many *Astragalus* species) then remain the species’ natural means of protection. If disturbances are too weak, networking concepts for habitats (e.g., NATURA 2000 for Europe) fail. However, if the natural linkage between habitats cannot be restored, networking is not necessarily desirable when speciation is in progress because it might be

thwarted by artificial hybridization or homogenization among populations. The protection of ecological processes is, therefore, highly recommended instead of pursuing a networking concept at all cost. As a consequence, conservation efforts must hastily be improved to protect the disjunct populations of *O. pilosa* and to retain their genetic diversity as a valuable natural resource.

**Acknowledgments** Special thanks go to Angela Dressel and Karin Stögerer for technical assistance, and to Wilhelm Sauer for discussion. Reiner Schmahl, Eberhard Koch, Natalie Schmalz and Matthias Kropf, Ludmilla Barjaewa, Ludmilla Mantschenkowa, and Erich Kalmbach, Friedrich Schlee, Evgenia Zeidler-Schewzowa, and the University of Barnaul (Altai, Siberia) are kindly acknowledged for contributing plant material, GZU, LY, M, and MA for sending specimens for revision. Hospitality of the visited herbaria BC, BOLO, C, DR, E, FB, FI, FR, G, HBG, K, LE, LI, LJU, M, MPU (incl. SIGMA), P, RO, S, STU, TUB, UPS, W, WU, and Z was enjoyed with great pleasure. The project was supported by a grant of the “Landesgraduiertenförderung Baden-Württemberg”; grants for travelling were obtained from “Reinhold-und-Maria-Teufel-Stiftung” and the German Academic Exchange Service (DAAD).

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# Relict Populations and Endemic Clades in Palearctic Reptiles: Evolutionary History and Implications for Conservation\*

Ulrich Joger, Uwe Fritz, Daniela Guicking, Svetlana Kalyabina-Hauf, Zoltan T. Nagy, and Michael Wink

**Abstract** The phylogeographic history of eight species complexes of West Palearctic reptiles was reconstructed using mitochondrial and nuclear markers. Cryptic endemic taxa were detected in the Southern European peninsulas (Iberia, Southern Italy/Sicily, and Greece) as well as in North Africa, Anatolia, Iran, and the Caucasus. These endemics are mainly of Tertiary or early Pleistocene age. Only part of them can be categorized as relicts of a former, more widely distributed taxon, having survived in ice age refugia; others have probably always remained in a restricted area, close to their origin.

Typical Pleistocene relict populations are phylogenetically related to larger conspecific populations elsewhere. They may be restricted to mountain habitats like *Vipera berus* in the Alps and in Greek mountains, or to islands like *Lacerta bilineata* on Cres and *Natrix tessellata* in Crete.

Holocene relict populations stem from a postPleistocene range expansion (usually in Southeast–Northwest direction). They reached their largest distribution during the Holocene climatic optimum and were later restricted to small areas in the climatically most favorable regions. Examples are *N. tessellata*, *Zamenis longissimus*, *L. bilineata*,

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\* We dedicate this chapter to the memory of Peter Lenk (1964–2005)

*L. viridis*, and *Emys orbicularis* in Germany. These populations are only poorly differentiated genetically; hence the risk of losing genetic diversity is not an appropriate argument for their protection. They may react to global warming by a range expansion.

Southern European, North African, and Middle Eastern relicts may, on the contrary, be threatened by global warming. These populations are in need of more studies, and should be brought to the attention of national and international conservation agencies.

## 1 Introduction

Reptiles are known as excellent indicators of the biogeographical history of a given region. This is due to their limited mobility (no long-distance dispersal), effectiveness of ecological barriers, and strong reaction to climate change due to ectothermic metabolism. The same three factors are also effective in isolating populations over a shorter or longer time; thus relict populations may be frequently isolated, and where favorable conditions prevail, they may survive up to the present. Dependent on the isolation time, separate populations develop genetic differences, but particular populations may also retain ancestral character states while other ancestral populations have gone extinct.

The importance of conserving genetic diversity is an internationally accepted goal since the 1992 Rio conference. With this in mind, molecular phylogeographic analyses serve two goals:

1. To reconstruct the evolutionary history of a group of organisms
2. To explore the spatial distribution of genetic diversity with special focus on unique genotypes restricted to particular regional populations; these populations deserve special attention in conservation programs.

A general requirement for a serious phylogeographic study must be that it should cover (ideally) the whole geographic range of a given species complex (Avice 1994).

The results summarized here have been obtained during a large-scale research project to elucidate the phylogeography of eight European reptile species complexes (overviews in Joger et al. 2006, 2007): The European pond turtles (*Emys orbicularis* complex), the water snakes of the genus *Natrix*, the European whip snake (*Hierophis viridiflavus*), the Aesculapian snakes (*Zamenis longissimus*/*Z. lineatus*), the adders (*Vipera berus* complex), the green lizards (*Lacerta viridis*/*L. bilineata*), and the sand lizard (*L. agilis*). These species complexes were selected so that similar phylogeographic patterns can be expected.

## 2 Materials and Methods

### 2.1 Laboratory Techniques

Blood samples were taken in the field (or from captive specimens of known collection locality) by caudal puncture (see Joger and Lenk 1997). The animals were released after photographic documentation. DNA was also extracted from ethanol preserved

tissues. We used a standard method for obtaining total genomic DNA (Sambrook and Russell 2001).

As a gene tree is not necessarily identical to the species tree (see Avise 1994), we analyzed both mitochondrial and nuclear markers, wherever possible. Mitochondrial DNA, predominantly cytochrome b, was amplified by PCR and directly sequenced. (see the cited original publications for primer sequences). The applied nuclear markers were either allozymes and plasma proteins, or the inter-spaces between microsatellites – ISSRs (see Joger et al. 2006, 2007).

Both kinds of markers supplement each other and their combination thus enhances the capacity to resolve the phylogeographical history. Mitochondrial sequences are powerful tools to reconstructing subsequent multiple splitting events. Moreover, as they are inherited only maternally, without recombination, introgressions lead to the coexistence of several matriline (haplotypes), thus preserving multiple origins of populations. On the other hand, hybridization (e.g., in secondary contact zones) cannot be detected by mitochondrial DNA. Bisexually inherited nuclear markers do not have this restriction.

## 2.2 Phylogenetic Reconstruction

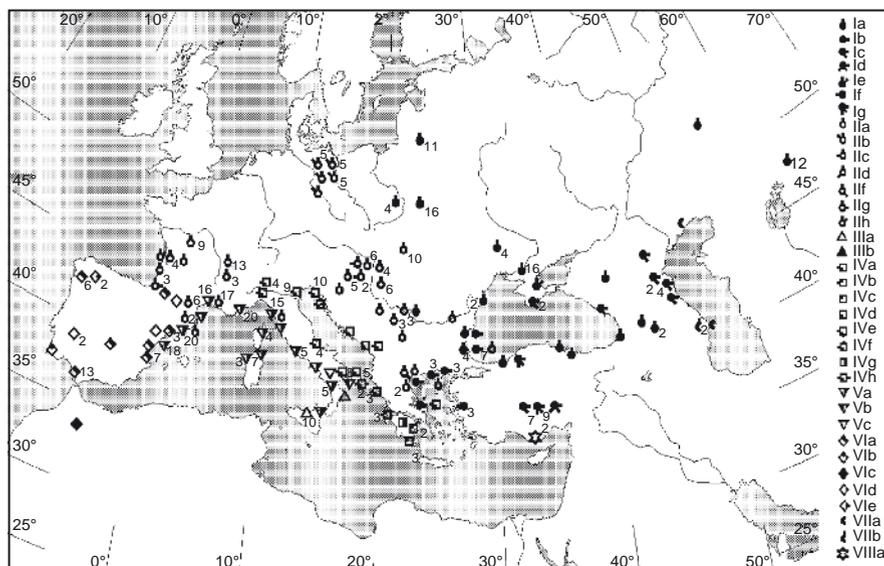
For phylogenetic reconstruction from the cytochrome b sequences, three methods were employed – maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference of phylogeny (BI). MP and ML analyzes were used as implemented in the program Paup 4.0b10 (Swofford 2002). Bayesian phylogenetic analysis was performed using the program MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001). Because prior analyzes have shown that saturation effects are negligible in our data set, all characters were equally weighted in the parsimony analysis. ML trees were calculated using the models selected under the Akaike Information Criterion (AIC) in Modeltest 3.06 (Posada and Crandall 1998), using heuristic searches with tree-bisection-reconnection branch swapping and ten random addition sequence replicates. Bayesian analysis was performed in four chains for 1–2 millions of generations with the same parameter settings as for the ML calculation.

Support for clades from both data sets was assessed by bootstrapping (Felsenstein 1985). Bootstrap analyzes for neighbor joining and MP were performed with 1,000 replicates and under the same parameter settings as for the original trees. Under the ML criterion, bootstrap analyzes were performed with 100 replicates and without branch swapping to save computation time.

## 3 Results

### 3.1 European Pond Turtle (*Emys orbicularis* Complex)

*Emys orbicularis* (Linnaeus, 1758) is a polytypic species composed of a number of morphologically defined subspecies (Fritz 1996).

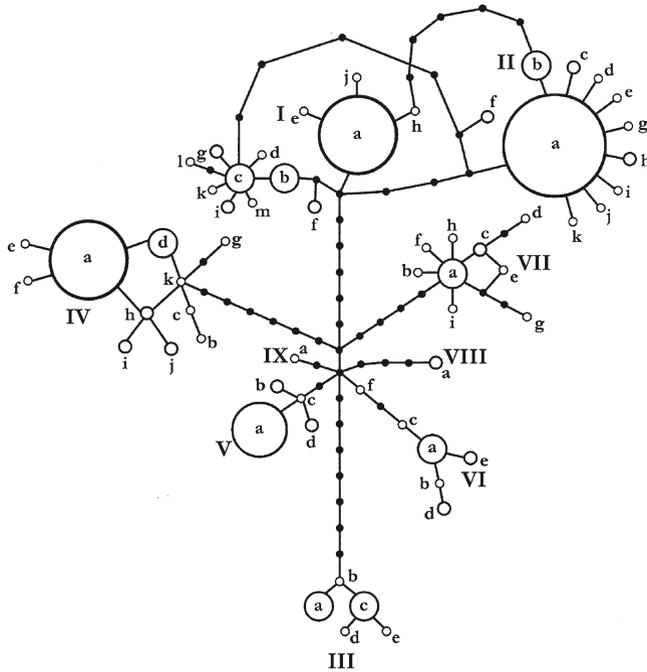


**Fig. 1** Geographical distribution of *Emys* haplotypes (only natural populations). Some rare haplotypes are not shown. Note that only haplotypes of groups I and II occur naturally North of the Alps

We sequenced the mitochondrial cytochrome b gene of more than 1,100 individuals of pond turtles from about 200 localities, and identified 56 different haplotypes (see Fritz et al., 2007). These could be grouped into nine monophyletic haplotype groups (Figs. 1 and 2).

Group I is composed of ten haplotypes (Ia to Ij). They are distributed in Eastern Europe, in the Aegean region and in Anatolia. Lineage If from Izmir province seems to represent the most ancient surviving haplotype. Only haplotype Ia is found North of the Black Sea and the Crimea – all others occur further South. The considerable variation of group I in Turkey points to the possibility of several refugial areas in the Anatolian peninsula. From there, postglacial expansion most likely occurred both eastwards and westwards, around the Black Sea.

Group II is the sister group of group I. Both constitute the nominate subspecies *E. o. orbicularis*. Group II occupies an arc-shaped range from Northern Greece across Danube lowlands, parts of Central Europe, and France to North-Eastern Spain. The haplotype with the widest range- Iia- is the direct ancestor of all other haplotypes II, except the more ancestral haplotype IIf which is found in Macedonia. This indicates a possible Pleistocene refugial area in the Southern Balkan Peninsula. From there, haplotype Iia spread along the Danube valley, westwards North of the Alps (see Fritz 1996). Haplotype Iib, found in Eastern Germany only, derived from Iia by one mutational step like other local haplotypes (see Fig. 2). The question whether small German populations of haplotype Iia are indigenous relicts or



**Fig. 2** TCS parsimony network of the *Emys* haplotypes. Haplotype group III=*Emys trinacris*. Open circles symbolize known haplotypes, circle size corresponds to the frequency of haplotypes as detected; dots on lines show single substitution steps between haplotypes (most parsimonious connection)

derived from introduced individuals, is debated among conservationists (see Fritz et al. 2004).

Group III inhabits Sicily and possibly the extreme South of the Italian mainland. As this group is highly differentiated from all others both genetically and morphologically, it has been described as a separate species, *E. trinacris* (Fritz et al. 2005). It must have remained in isolation for a long time.

Group IV is found around the Adriatic Sea, from Southern Greece to Italy, East of the Apennines. The Northernmost parts of the range are occupied by haplotype IVa only. On the island of Evvia, haplotypes IVa and Ib occur together.

Group V replaces group IV West of the Apennines. It reaches around the coast of the Tyrrhenian Sea to Catalonia and also inhabits Sardinia and Corsica. In Northeastern Spain it occurs in mixed populations with haplotypes IIa, VIa, and VIc, in Apulia with IVb and IVd, and in Sicily with IIIa.

Group VI is confined to the Iberian Peninsula and Northwestern Africa. Its most ancestral haplotypes, VIc, and VIf, are found in Morocco. North Africa should therefore be considered as the probable origin of group VI.

Group VII is only found in the Caspian area and Iran, and group VIII is restricted to Southern Anatolia. The geographic origin of group IX is unknown.

### 3.2 Green Lizards (*Lacerta viridis*/*L. bilineata*)

*Lacerta viridis* has been subdivided into two biological species, Western European *L. bilineata* Daudin, 1802 and Eastern European *L. viridis* (Laurenti, 1768). The hybrids between them show reduced fertility (Rykena 1991, 1996, 2001)

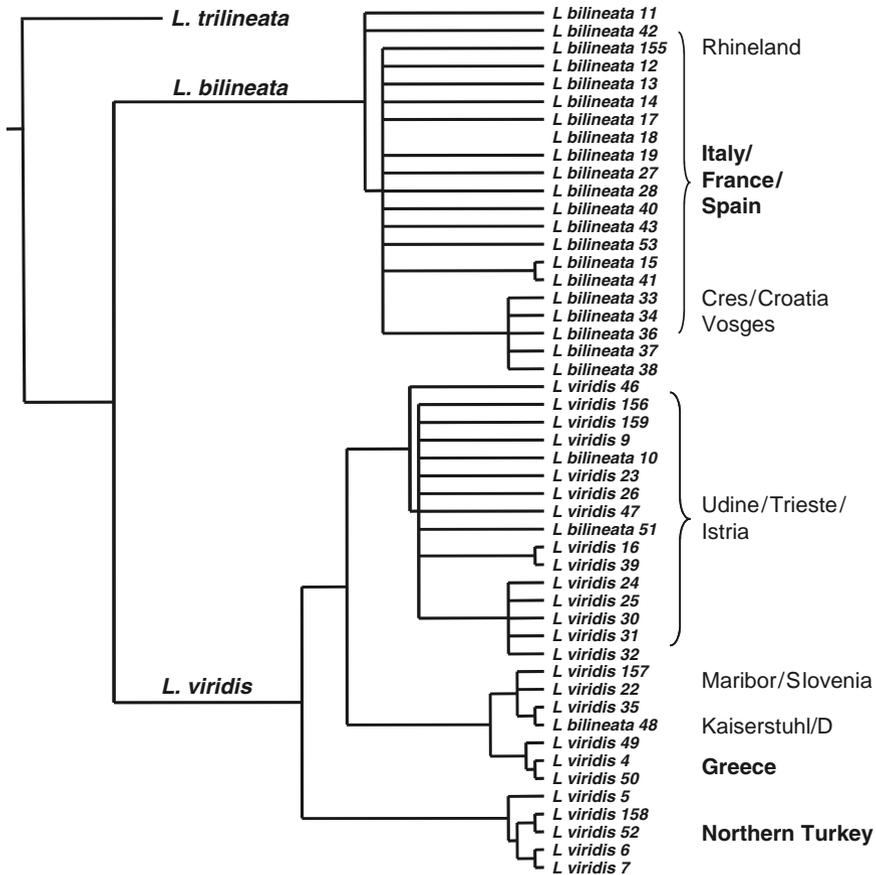
Amann et al. (2001) identified a hybrid zone in Northeastern Italy and the adjacent part of Slovenia, in which a restricted and asymmetrical gene flow (predominantly from the East to the West) could be detected (Joger et al. 1998). This type of hybrid zone of “species in statu nascendi” will remain spatially restricted, but may be stable for a long time, if it is stabilized by equivalent invasion and evasion, hybridization, and selection (Barton and Hewitt 1988).

Our cytochrome b tree (Fig. 3) is concordant with a previously published allozyme tree (Amann et al. 1997, Joger et al. 2001) by grouping in two main clusters – the two species *L. bilineata* and *L. viridis*. Both trees group the populations from the hybrid zone (Udine, Trieste) to *viridis*, which contradicts a recent analysis by Böhme et al. (2006). These authors included those populations (and Slovenian populations) in a “Western Balkan group” extending South to Greece. As this group clustered with *L. bilineata* in their tree, they assigned it to *L. bilineata*, in order to prevent a paraphyletic *viridis*. Mayer and Beyerlein (2001), using 12s and 16s RNA genes, also found Western Greek *viridis* associated with *bilineata*, while Brückner et al. (2001) found that cytochrome b from the same animals clustered with *viridis*. These contradictory results were confirmed by Godinho et al. (2005). However none of the teams had included Turkish *L. viridis* in their analysis. In our tree (Fig. 7), with Turkish lizards included, there is no paraphyly of *L. viridis*. The lizards from Trieste and Udine cluster with *viridis*, as well as the Western Balkan group. The Croatian population from the island of Cres clusters with the Western *bilineata*. This was also found by Brückner et al. (2001) and by Godinho et al. (2005). Cres may harbor a relict population of a once more easterly distributed *bilineata*, recently restricted by a Western advance of *viridis*.

For both *L. viridis* and *L. bilineata*, a number of subspecies have been described (Nettman 2001). Most of them are found in Southern Italy (*L. bilineata*), Greece, or Turkey (*L. viridis*) and point to multiple glacial refuges in those areas. However, the molecular data are not yet sufficient to decide upon this issue.

### 3.3 Sand Lizards (*Lacerta agilis* Complex)

*Lacerta agilis* Linnaeus, 1758, is a widespread species in Europe and Northwestern Asia. Nine subspecies have been described (Bischoff 1988). A phylogeographic analysis based on cytochrome b sequences (Kalyabina et al. 2001, Kalyabina-Hauf et al., 2004a) confirmed most of the subspecies, and revalidated a 10th one, *L.a.tauridica* Suchov, 1926, from Crimea. If the branching events (Fig. 4) are linked with the geographical distribution of the subspecies, the Caucasian/Black Sea region

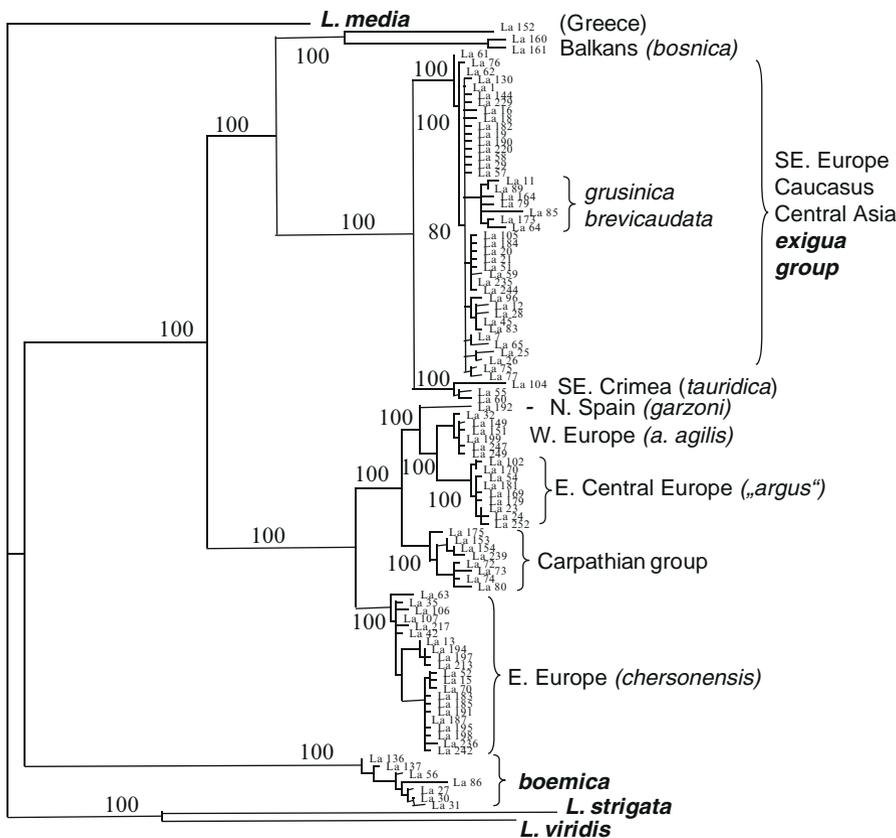


**Fig. 3** Maximum parsimony tree for Green lizards, derived from mitochondrial cytochrome b sequences. Note that several individuals were initially determined as *L. bilineata* (from Trieste, Italy, and Kaiserstuhl, Germany), but have haplotypes of *L. viridis*, whereas lizards from the Croatian island of Cres are unambiguously *L. bilineata*

emerges as the group’s most probable area of origin (Joger et al. 2007). The most ancestral surviving clade is *L. a. boemica* Suchov, 1929, from the Eastern Caucasus. It may deserve species status.

The Western clade (distributed from Western Russia to the Pyrenees), in which the earliest internal branches lead to the easternmost subspecies – *L. a. chersonensis* and the next branching to an unnamed Carpathian group – must have moved gradually westwards.

The Eastern clade (from the Caucasus West to Crimea and East to Lake Baikal), and a Balkan subspecies (*L. a. bosnica*) are the other two major clades. As *L. a. bosnica* is sister of the Eastern clade, while the Western clade branched off from the common stem earlier, the most probable phylogeographic hypothesis must take into account several waves of colonization from East to West. A molecular



**Fig. 4** Maximum Parsimony phylogram (consensus of 300 equally parsimonious trees) of the *Lacerta agilis* complex, derived from mitochondrial cytochrome b sequences. *L. media*, *L. strigata*, and *L. viridis* are used as outgroups. Bootstrap values indicated. The *L. exigua* group unites subspecies from Caucasus (*L. a. grusinica*, *L. a. brevicaudata*) as well as Southeastern European *exigua* s.str. and various central Asian *agilis*

clock places these waves into different periods, from upper Pliocene to middle Pleistocene. The colonization of Central Asia appears to be more recent (maybe even Holocene).

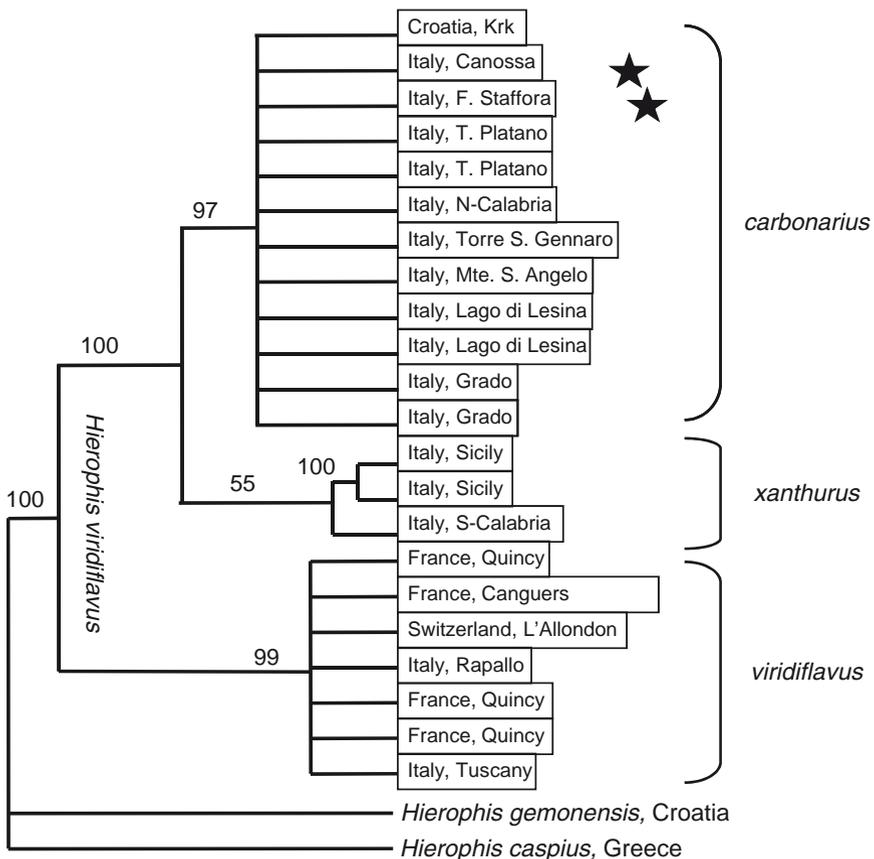
### 3.4 European Whip Snake (*Hierophis viridiflavus*)

*Hierophis viridiflavus* (Lacépède, 1789) is distributed over most of France, Italy, and Slovenia and reaches into South-Western Switzerland, Luxemburg, Belgium, Northernmost Spain, and Croatia. An Eastern subspecies, *H. v. carbonarius* (Bonaparte, 1833), was mainly based on coloration and refuted by Schätti and Vanni (1986). However,

our molecular analysis based on both cytochrome b sequences and ISSR fingerprints, confirmed it (Nagy et al. 2002).

In general, a Western and an Eastern clade are discernable, the border between them being the Apennine chain (Fig. 5). A third clade, found in Sicily and Southern Calabria, is sister to the Eastern clade. Its subspecific name should be *H. v. xanthurus* (Rafinesque, 1810). ISSR fingerprints revealed distinctive loci for these three clades, but also identified several specimens of hybrid origin which share bands characteristic of Eastern and Western clade.

In this species, the phylogeographic history is also documented by fossil records from Austria, Southern Germany, Czech Republic, and Poland (Szyndlar and Böhme 1993, Ivanov 1997). These areas were reached in warmer periods of the Pleistocene, as well as in the Pliocene, but in glacial periods the distribution area



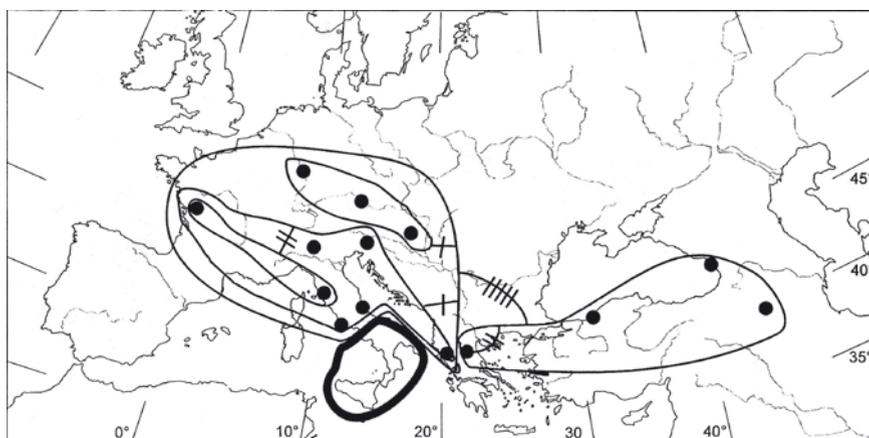
**Fig. 5** Phylogeography of *Hierophis viridiflavus* derived from cytochrome b sequences. Maximum Parsimony tree with bootstraps values for the main haplotype groups. \*=Populations East of the Apennines, in which alleles of the Western group were recorded by ISSR-PCR (putative hybrids)

shrunk to its Italian core area where the genetic differentiation occurred. No relicts remained outside the main distribution area.

### 3.5 Aesculapian Snake (*Zamenis longissimus* Complex)

*Zamenis longissimus* (Laurenti, 1768), formerly *E. longissima*, is found in France, Italy, Austria, the Balkans, and Turkey, with isolated relict populations in Southern Germany, Czech Republic, and Poland.

Protein electrophoretic comparisons (Lenk and Joger 1994) revealed only slight geographic structuring in *Z. longissimus* which, for example, showed an association of the Central European relicts to the Balkan populations and not to the Italian populations (thus refuting the hypothesis that the Romans had imported the Aesculapian snake to Germany). Moreover Peters (1977) had already reported fossil *Z. longissimus* from deposits of the early Holocene climatic optimum in North-Eastern Germany. However, a strong difference in blood protein electrophoretic patterns was found between Southern Italian Aesculapian snakes and all others. A morphological analysis (Lenk and Wüster 1991) concluded that the Southern Italian population must be regarded as a separate species, *Z. lineatus* (Camerano, 1891). A cytochrome b sequence analysis (Fig. 6) also shows the external position of *Z. lineatus* with regard to *Z. longissimus* (sequence difference of 7%). Moreover, it shows that in *Z. longissimus*, an Eastern haplotype group (found along the Aegean and Black Seas) can be distinguished from a Western haplotype group. Both groups nearly meet in Greece. They are separated by the Pindos Mountains and must have had different Pleistocene refugia. The Western haplotype group is composed of a Danubian haplotype (which includes the German and Czech populations) and a Mediterranean haplotype, from which a Western haplotype (hitherto found in

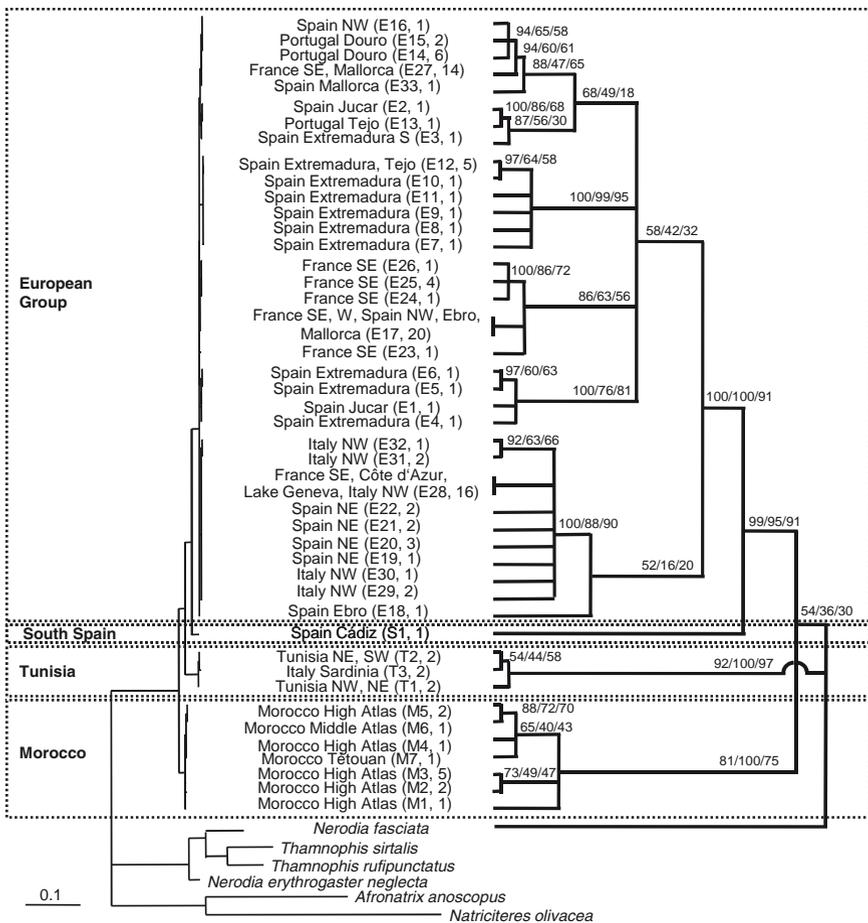


**Fig. 6** Phylogeographic grouping of Aesculapian snakes (*Zamenis longissimus* and *Z. lineatus*) derived from mitochondrial cytochrome b sequences

France and in Tuscany) derived. Three microrefuges may therefore be assumed for the Western haplotype group.

### 3.6 Viperine Snake (*Natrix maura*)

*Natrix maura* (Linnaeus, 1758) inhabits aquatic habitats in the Western part of the Mediterranean, from Italy to Morocco and Tunisia, North to Central France, and Lake Geneva. Two phylogeographic analyzes were done on the basis of cytochrome b sequences (Barata et al. 2008, Guicking et al. 2008) and ISSR fingerprints (Guicking et al. 2008). Both data sets showed identical groupings (Fig. 7).



**Fig. 7** Phylogenetic trees of *N. maura* reconstructed from cytochrome b haplotypes, *left*: maximum likelihood phylogram, *right*: Bayesian cladogram. Sequences from *N. natrix* and *N. tessellata* have been used for outgroup rooting. Support values are indicated in the order Bayesian/ML bootstrap values/MP bootstrap values

3 major haplotype groups were found – two North African (Tunisian, Moroccan) and one European. Eastern Moroccan and Algerian *Natrix* cluster with the Tunisian group (Barata et al. 2008). The origin of the species can be assumed in North Africa. The fact that one of the Tunisian haplotypes is also found in Sardinia may be explained by human introduction, probably in Roman times. The Viperine snakes of Mallorca were probably introduced, too, as they share a haplotype which also occurs on the French and Spanish mainland (Guicking et al. 2006b).

In the European group, a haplotype from extreme Southern Spain is sister to all other European haplotypes. It may therefore be regarded a relict of the first European settlement of *N. maura*. A standard molecular clock dates the colonization of the Iberian Peninsula as Pliocene (ca. 3 m.y.b.p.). A comparable phylogeographical pattern exists in the turtle *Mauremys leprosa* (Fritz et al. 2005).

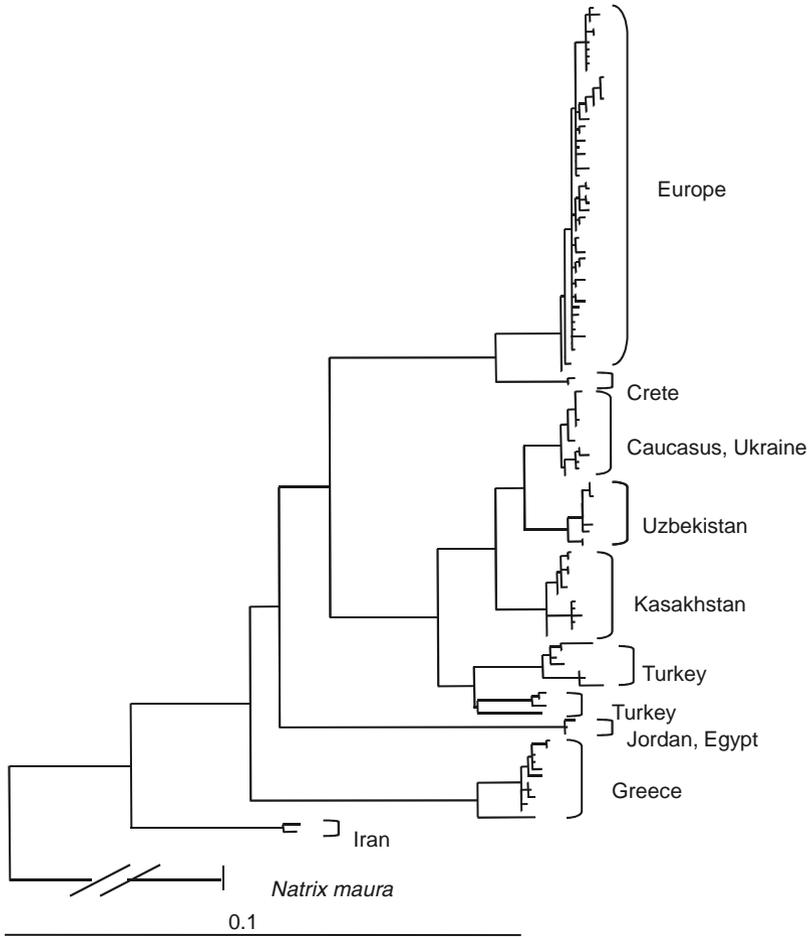
In Europe, genetic diversity is lowest among Northeastern populations (Italy, France, Switzerland, NE Spain), while distinct genetic groups occur in Southern Spain and Portugal (Extremadura, Tejo). This is paralleled by other Iberian taxa, such as salamanders (Joger and Steinfartz 1994). Southern Iberian populations are more isolated and most differentiated. Northern Iberian populations are the sources of post-Pleistocene colonizations of France and Italy, but an additional micro-refuge in Southern France or Western Italy cannot be excluded.

### 3.7 *Dice Snake (Natrix tessellata)*

*Natrix tessellata* (Laurenti, 1768) is the sister species of *N. natrix* (Guicking et al. 2006a). The Dice snake is the ecological equivalent of the Viperine snake further East, occupying a vast distributional range from Germany, Switzerland, and Italy to Central Asia, Iran, and Arabia. The phylogeographic analysis of cytochrome b sequence data revealed nine distinct haplotype groups, of which two occur in Greece and only one in the rest of Europe. The Middle East is the most probable area of origin of this species. Early branching events led to highly differentiated haplotype groups found in Iran (up to 10% sequence difference), Greece, and Northern Arabia (Figs. 8 and 9). Four haplotype groups occurring around the Black Sea in Turkey, and Central Asia are related to each other; ISSR fingerprints show that populations of these groups hybridize. A distinct group is found in Crete, and a rather homogeneous group occupies all the European range except Greece.

The existence of two separate Pleistocene refugial areas in the Southern Balkans can be deduced from the data. Only one of them served as a source for the recolonization of the rest of the European area. The genetic diversity among European populations is low and shows little geographic structuring. The invasion of Italy could be dated back to the last interglacial maximally, and thus a separate microrefuge in the Apennine peninsula is possible for the last glacial (Guicking et al. 2009).

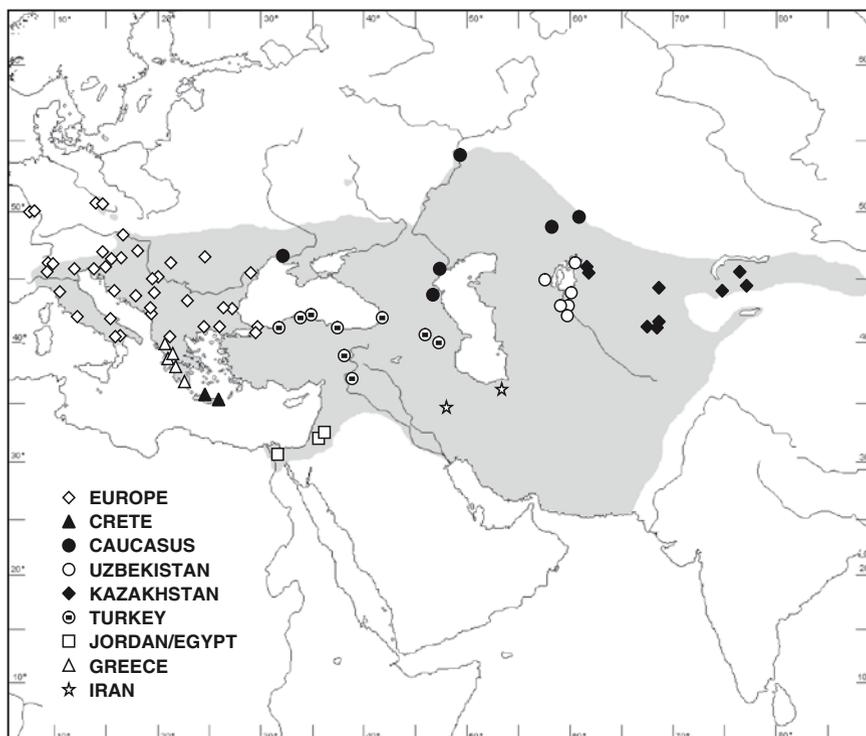
A number of microrefuges must have existed in Anatolia, in the Caucasus, and in West Central Asia.



**Fig. 8** Maximum Likelihood Phylogram for *Natrix tessellata*, based on complete cytochrome b sequences. 9–10 different haplotype groups can be distinguished. *Natrix maura* served as outgroup

### 3.8 Adders (*Vipera berus* Group)

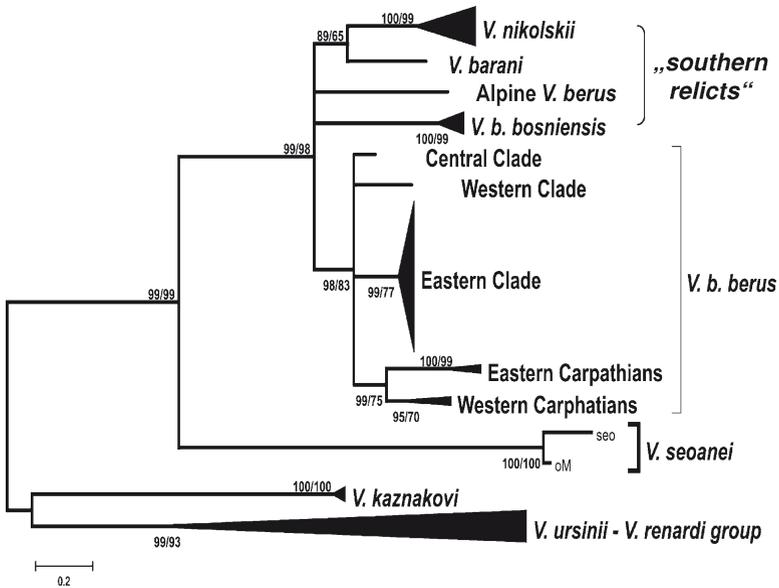
In contrast to the species treated earlier, adders have a more Northern distribution, occupying a vast territory from Northern Spain to the Polar circle in Scandinavia, and to the Pacific Ocean in the East. Yet here too, genetic differentiation and speciation are concentrated in the Southern margin of the distribution area. The Iberian Adder, *V. seoanei* Lataste, 1879, has been regarded as a separate species for long. In the Black Sea area, *V. nikolskii* Vedmederya et al. 1986 (Ukraine, Southern Russia) and *V. barani* Joger and Böhme 1984 (Turkey) have been described, but their species status is doubtful. In the Balkans, a subspecies *V. berus bosniensis*



**Fig. 9** Geographical distribution of haplotype groups in *Natrix tessellata*, modified from Guicking et al. (2009.)

Boettger, 1889, is known, and in the Far East, *V. berus sachalinensis* Tsarevski, 1916, has been distinguished. The relationships between these taxa have been studied by Kalyabina-Hauf et al. (2004b) and by Ursenbacher et al. (2006a).

In the cytochrome b tree (Fig. 10), *V. seoanei* appears as a sister to all other adders. *V. nikolskii* and *V. barani* cluster into one clade, though not with strong support. The genetic separation of Alpine *V. berus* from the rest of the species points to a separate glacial refuge South of the Alps (probably in Italy, where a population of lowland adders became extinct just several decades ago [see Scali and Gentilli, 1999]). It is also supported by ISSR fingerprint data (Kalyabina-Hauf et al. 2004b) and by mitochondrial control region sequences (Ursenbacher et al. 2006a). The refugia of Northern *berus* are difficult to determine, but the separate position of the Hungarian samples points to the Carpathian basin as one possible refuge. According to Ursenbacher et al. (2006a), additional refugia may have been located in France, Slovakia, or Southern Russia. Scandinavia was recolonized post-glacially from two sides, West and East, so several glacial refugia to this fairly cold tolerant snake are reasonable.



**Fig. 10** Maximum Parsimony strict consensus tree of *Vipera berus* and relatives derived from cytochrome b haplotypes (1,003 bp). Bootstraps values indicated for Maximum Parsimony and Maximum Likelihood. *Vipera kaznakovi*, *V. renardi* and *V. ursinii* were used as outgroups

## 4 Discussion

### 4.1 Phylogeographic Patterns

The degree of genetic differentiation is fairly high in all studied species complexes, regardless of morphological variability. A species like *E. orbicularis*, which has a high number of geographically restricted morphological subspecies, shows a similar amount of genetic variation as *N. tessellata* and *N. maura* – species that have not been subdivided previously, due to morphological uniformity. These three species are more or less aquatic.

A general observation in all species complexes is the fact that genetic variation is geographically biased: It is generally lower in the North and much higher in the South. This reflects the effects of climatic change during the Pleistocene: Northern territories were invaded during interglacial periods (such as the present one), but the newly acquired territories had to be given up when the next glacial advanced (Taberlet et al. 1998). Thus in most taxa, today’s occupants of the areas North of the Alps have a short history: The molecular trees show that their ancestor came from Southern refugia after the end of the last glaciation, i.e., about 10,000 years ago. This period was obviously too short for major genetic changes; only some private haplotypes, differing by a few mutations, have been found in the Northern territories (such as haplotype IIb in Central European *E. orbicularis*).

Moreover as a rule, only one or two of several existing haplotype groups of any given species managed to conquer the Northern part of the species' territory, and these haplotype groups also persist in their putative area of origin further South.

There are a few exceptions to this rule. In *V. berus*, the Northern part of the range is occupied by a typical haplotype group that does not occur South of the Alps. It has however, a wide distribution to the East, reaching the Pacific coast of Asia. Therefore, although it is a boreal species, it probably did not survive the last glacial in Northern Europe, but rather in Eastern Europe or Asia, where a number of tree species had their refugia too (Gliemerth 1995).

Another case is *L. agilis*, which is not a Mediterranean species. Its evolutionary center can be identified in the Caucasus. There are a few distinct Northern haplotype groups, two of them in Central Europe. *L.a.agilis*, the Western group, extends from South-central France to Western Germany, whereas *L.a.argus*, its Eastern sister, is found from Eastern Germany to Romania. This pattern parallels the distribution of *L. bilineata* (West) and *L. viridis* (East). Both taxon pairs may have had two glacial refugia: one in the Southeast and one in the Southwest, and invaded Central Europe from both sides simultaneously. The difference lies in the degree of speciation. The Central European *L. agilis* subspecies are quite young and may be interpreted as a product of the latest glacial fluctuations, whereas *L. viridis* and *L. bilineata* have had time to proceed much further in the speciation process. Their genetic differentiation must have started in the early Pleistocene (Joger et al. 2007).

## 4.2 The Time Scale of Evolution

The average evolutionary rate for Viperidae cytochrome b has been estimated to 1.4% sequence difference per 1 million year (Ursenbacher et al. 2006a). For *Natrix*, we calculated a similar rate of 1–1.35% per 1 million year (Guicking et al. 2006a). For *L. agilis*, we estimated a higher rate of up to 2.5% per 1 million year (Kalyabina et al. 2001), but for *E. orbicularis*, a lower level of 0.3–0.4% per 1 million year (Lenk et al. 1999). These differences are interpreted as reflecting the different metabolic activity in reptiles, which is lowest in turtles and highest in lizards. Life expectancy is reciprocal to metabolic rates.

If these estimates are correct, sequence differences of 1% or more in turtles, 3% or more in snakes, 6% or more in lizards are due to prePleistocene branching events and hence, cannot be associated with Ice Age refugia. All species groups investigated here originated in the Tertiary and speciation events are of Pliocene age (Table 1). This is in accordance with the fossil record, as remains of today's snake species were found in Pliocene sediments as old as 3.4 Million years (Szyndlar and Böhme 1993). A mid-Miocene record of *Natrix* was considered a direct ancestor of the extant *N. natrix*, suggesting that the three clades in *Natrix* might have already been separated in the middle Miocene (Ivanov 2001). According to our estimate, the main clades of *Natrix* species are of late Miocene age, the Southern Spanish clade of *N. maura* is dated Pliocene, and the differentiation between the lineages of the main

**Table 1** Speciation, sequence differences and approximate time estimates

Group	% sequence difference between sister species (cyt b)	Time estimate for species (m.y.)	Time estimate for major groups within species (m.y.)	Reference
<i>Emys</i>	1.4–1.7 <sup>a</sup>	3–4	1.4–3	Lenk et al. (1999)
<i>Lacerta agilis</i>	6.5–7.3 <sup>b</sup>	2.6–2.9	0.7–2.4	Kalyabina et al. (2001)
<i>L. viridis</i> complex	6.6–8.4 <sup>c</sup>	2.6–3.4	0.5–2.5	Böhme et al. (2006)
<i>Hierophis</i>	10.5 <sup>d</sup>	8–9	0.9–2.7	Nagy et al. (2002, 2004)
<i>Zamenis</i> ( <i>Elaphe</i> )	7 <sup>e</sup>	5–7	0.5–1	Lenk et al. (2001)
<i>Natrix</i>	18 <sup>f</sup>	13–22	2–8	Guicking et al. (2006a)
<i>Vipera berus</i> group	5 <sup>g</sup>	4	1.1–1.6	Kalyabina-Hauf et al. (2004b)

<sup>a</sup>Distance between *E. orbicularis* und *E. trinacris*

<sup>b</sup>Distance between *L. agilis* ssp. and *L. (a.) boemica* (assuming species status)

<sup>c</sup>Distance between *L. viridis* and *L. bilineata*

<sup>d</sup>Distance between *H. viridiflavus* and *H. gemonensis*

<sup>e</sup>Distance between *Z. longissimus* and *Z. lineatus*

<sup>f</sup>Distance between *N. natrix* and *N. tessellata*

<sup>g</sup>Distance between *V. berus* and *V. seoanei*

European clade is Pleistocene (Guicking et al. 2006a). On the other hand, Nagy et al. (2003) estimated evolutionary rates for colubrid snake mitochondrial genes as twice the rate as we assume here, hence, of the same magnitude as in *Lacerta*. If we accept this alternative, *Hierophis* and *Zamenis* would show similar time estimates for sister species as *Lacerta* (Pliocene); but *Natrix* speciation would still be Miocene.

Apart from *Natrix*, the main intraspecific clades in our selection are of late Pliocene or Pleistocene age. As pronounced climatic oscillations started already in late Pliocene, it seems appropriate to consider these intraspecific radiations as effects of climate change and associated range restrictions.

### 4.3 Pleistocene Refugia and Recolonization

Table 2 gives an overview of Pleistocene refugia inferred for the studied groups. The classical Mediterranean refugia (Iberia, Italy, and the Balkans) were used by all species groups, but in many cases, more than one microrefugium was located in each peninsula.

North African and Asian refugia did not play a role in recolonization after Pleistocene cold phases, but have acted as long time independent speciation centers. This observation may however, be biased by our selection of species, as we took into account only species which were able to colonize Central Europe (at least its climatically most favorable regions).

**Table 2** Pleistocene refugia inferred from cytochrome b haplotype partitions

Group	Iberian	Apennine	Balkans	Pontic	Caspian	Other
<i>Emys</i>	several	several	several	several	1	North African
<i>Lacerta agilis</i>	1	–	2	1	several	Carpathian
<i>L. viridis</i> complex	–	2	several	1	–	
<i>Hierophis</i> <i>viridiflavus</i>	–	several	–	–	–	
<i>Zamenis</i> <i>longissimus</i>	–	2	2	?	?	
<i>Natrix maura</i> / <i>N. tessellata</i>	several	?	2	several	several	North African West Asian
<i>Vipera berus</i> group	1( <i>V. Seoanei</i> )	1	1	2	–	Carpathian French

Among these species, the two most cold-tolerant, *V. berus* and *L. agilis*, were probably able to survive in non-Mediterranean refuges like central France and the Carpathian basin. A glacial refuge in France was also inferred for two species related to the aforementioned, *V. aspis* (Ursenbacher et al. 2006b) and *L. (Zootoca) vivipara* (Guillaume et al., 2000). A glacial refuge in the Carpathian basin has been assumed for two species of considerable cold tolerance, the Moor frog *Rana arvalis* (Babik et al. 2004) and the Field vole, *Microtus agrestis* (Jaarola and Searle 2002).

In genera which had refugia in all three European peninsulas (*Emys*, *Natrix*), four paradigms for post-glacial range expansion are possible (Hewitt 1999, 2000; Habel et al. 2005). Yet only one of them was used: The Eastern refugia in particular were the source areas for the colonization of Northern areas such as Germany. The reasons for this may be both geographical and ecophysiological. The lack of mountain barriers in Eastern Europe allowed a quick range extension once the climatic situation became favorable. On the other hand, Eastern refugia had a continental climate with more severe winters than Western refugia. Populations adapted to that climate could start their Northward expansion earlier and reach more Northern locations. In *E. orbicularis*, the Balkan-originated haplotype II crossed Central Europe North of the Alps and France to Northeastern Spain, while the Western Mediterranean haplotypes stayed where they were. This is paralleled by the Eastern shrew *Sorex araneus*, which reached Northeastern Spain post-glacially, but had to retreat into montane refuges there while the warmer climate favored the Western species *S. coronatus* (Lopez-Fuster and Ventura 1996).

#### 4.4 What is a Relict Population?

Generally, a relict population can be defined as a part of an evolutionary lineage which has been isolated geographically.

Three types of relicts have to be considered:

1. *Geographical relicts* without significant genetic differentiation. This type of relict is usually found in “exclaves” beyond the Northern margin of a species’ range. Typical representatives of this type are the minute German and Czech populations of *N. tessellata* and *Z. longissimus*. Both occupy geographically restricted, climatically favorable habitats along river valleys, preferably the South-facing (sunny) banks and adjacent hills of rivers flowing in latitudinal direction. The same areas are occupied by other “submediterranean” relict species such as *L. bilineata* and *Podarcis muralis*. Other such relicts (*L. viridis*, *E. orbicularis*) are found in sub-continental areas of Eastern Germany, which have more insolation in summer. All these species must have reached their presently isolated Northern territories in post-glacial times. In climatically favorable times, such as the climatic optimum 5,000–7,000 years ago, they must have extended their range to a much larger territory (e.g., *Z. longissimus* reached the Baltic Sea Coast (Peters 1977)). Yet the territories they occupy today are small and isolated. They are classified as *Holocene relict populations* (Table 3). Due to their very restricted occurrence and low population sizes today, these populations must be considered true relicts in Germany, and they are consequently classified in the highest national conservation categories. Yet, if the available genetic data are considered, their extinction would not mean significant loss to the species’ total genetic diversity. With the exception of the above-mentioned East German haplotype of *E. orbicularis* and some single mutations in other species, all major haplotypes found in the isolated German populations also occur in the larger territories of the respective species further South. Of course, caution has to be taken not to rely solely on a limited amount of genetic data – usually of mitochondrial genes. Theoretically, a wide genomic screening could change the observed pattern.
2. *Genetic relicts*: This type of relicts is usually found at the Southern or Eastern edge of a species’ range. Genetic relicts can be defined as possessing a unique, significantly differentiated haplotype or a haplotype group of their own. To qualify as a relict, a historical loss of territory is also necessary. Typically, these relicts have a long evolutionary history in one particular region and were unable to extend their range post-glacially. In some cases, such as the Alpine population of *V. berus*, the range probably shifted from lowland (Northern Italy) to highland (the Swiss and Austrian Alps), but the range extension to the North was accompanied by loss of territory in the South. This type of relict is older than Holocene. It is classified as *Pleistocene relict populations* (Table 3), if it is not considered taxonomically distinct. Pleistocene relict populations have often been isolated by ecological change (sometimes with human interference, as the aquatic *N. tessellata* in the desertified Aral Sea region, and *E. orbicularis* in North Africa). Others were isolated from the main distribution area by a competing species, as the *L. bilineata* population of Cres Island, surrounded by *L. viridis*, which is thought to have expanded its territory. Another case is *V. berus* in Greece, restricted to high mountain habitats by both climatic warming and competing *V. ammodytes*.
3. *Endemic relict taxa*: If the genetic isolation has prevailed over long enough time, a taxonomic recognition of the isolated population as an endemic subspecies or

**Table 3** Endemics and relicts among the investigated taxa

Meta-Taxon	Regional endemic subtaxa	Endemic relict subtaxa	Pleistocene relict populations	Holocene relict populations
<i>Emys orbicularis</i> complex	<i>E. trinaeris</i> Sicily	<i>E.o.persica</i> Caspian Area <i>E.o.ssp.</i> S. Anatolia	<i>E. orbicularis</i> North Africa <i>E.o.orbicularis</i> Turkey	<i>E.o.orbicularis</i> E. Germany, Lithuania, Kazakhstan
<i>Lacerta viridis</i> complex	<i>L. bilineata</i> ssp. S. Italy, Sicily <i>L. viridis</i> ssp. Balkans	<i>L. viridis</i> ssp. Turkey	<i>L. bilineata</i> Balkans, Cres Isl.	<i>L. bilineata</i> W. Germany <i>L. viridis</i> E. Germany
<i>Lacerta agilis</i> complex	<i>L.a.tauridica</i> Crimea	<i>L.(a.)boemica</i> Caucasus <i>L.a.bosnica</i> Balkans <i>L.a.garzonii</i> Pyrenees	<i>L.a.chersonensis</i> Carpathians	<i>L. agilis</i> Britain, Sweden
<i>Hierophis viridiflavus</i>	<i>H.v.xanthurus</i> S. Italy, Sicily			
<i>Zamenis longissimus</i> complex	<i>Z. lineatus</i> S. Italy, Sicily			<i>Z. longissimus</i> Germany, Czech Rep.
<i>Natrix maura</i>	undescribed North Africa	undescribed Andalucia	<i>Natrix maura</i> Southern Spain	
<i>N. tessellata</i>	undescribed Middle East	undescribed Greece, Middle East	<i>N. tessellata</i> Crete, Aral Sea region	<i>N. tessellata</i> W. Germany, Czech Rep.
<i>Vipera berus</i> complex	<i>V.b.bosniensis</i> Balkans	<i>V. barani</i> Turkey <i>V. nikolskii</i> Black Sea area <i>V. seoanei</i> Pyrenees	<i>V. berus</i> Alpine Region <i>V. berus</i> Greece <i>V.b.berus</i> Carpathians	

species may be justified. Many of these taxa may also be termed Pleistocene relicts. Table 3 shows some examples. A distinction must be made between an endemic relict taxon and an endemic taxon which is not considered a relict. This decision must be based on the phylogeographic position of the taxa. For example, *E. trinacris* is an early offspring of the Tertiary *Emys* population in Europe. It was isolated in Sicily, where it differentiated genetically, but there is no evidence that it ever had a larger range, or that it had descendants which migrated elsewhere. So it is an endemic but not a relict. On the other hand, *E. orbicularis persica* (group VII in Figs 1 and 2) and the Southern Turkish subspecies (group VIII) must have had a larger range in the past. They were probably linked to Mediterranean groups before their range was restricted by Pleistocene expansion of *E.o.orbicularis*. A comparable fate may be assumed for *L. viridis* ssp. from Turkey and *L. (agilis) boemica* from Caucasus. Their phylogenetic position at the base of their respective radiation suggests that these taxa remained in the area of origin of those species, where they survived in a small area only, while more derived subspecies successively occupied new territories. On the other hand, several *L. viridis* ssp. from Balkans and *L. agilis tauridica* from Crimea are endemics, but not isolated, and are not likely to have experienced shrinking of their range. In *Natrix*, a number of distinct haplotype groups have been identified at the Southern margin of the ranges of *N. maura* (Andalucia, North Africa) and of *N. tessellata* (Greece, Iran, Arabia), but no taxonomic consequences have yet been drawn from that observation. We can expect both endemics and endemic relicts among those undescribed taxa, which occur in or near the probable areas of origin of those species (Guicking et al. 2006a). Finally in the *V. berus* complex, relicts occur at the Southern margin of the distribution area of this cold-adapted vipers. *V. barani* is completely isolated in Turkey, and *V. nikolskii* has apparently lost territory by intrograding *V.b.berus* from the North (Zinenko, unpublished data). *V. seoanei*, retreating in the Pyrenees, may be under pressure from two warm-adapted species which occur parapatrically: *V. aspis* and *V. latastei* (Stümpel et al. 2005).

How will the expected change of climate (global warming) affect relict reptile species? In short, there will be advantages for relicts of thermophilic species in the North, which may enlarge their territories, as is already observed with some plants and invertebrates.

Pleistocene relicts in the South, now confined to mountain areas like *V. seoanei* in the Pyrenees and *V. berus* in the Greek mountains, will have to retreat to higher altitudes, while lowland species invade into their former territory. Relicts of European species in North Africa (*E. orbicularis*) or Asia Minor (*L. viridis*, *V. barani*) may die out, while other species take their place.

Thus future climate change will favor some species to the disadvantage of others.

#### 4.5 Conclusions for Conservation

Conservation priorities are set under national and international criteria. International goals were formulated at the Rio 1992 conference and include worldwide conservation

of genetic heritage. This would mean that Holocene relict populations – genetically still very close to their relatives in the main distribution area – deserve less attention than endemics, regardless of whether they are relicts or not. Pleistocene relicts would occupy an intermediate position.

Thus in international conservation strategies, PostPleistocene relicts can be neglected. However on a national basis, there is usually much emphasis on rarities such as Mediterranean relicts in Central Europe. Although they are common and not very cared about in the Southern part of their range, they attract much attention in the North. Moreover, they are “flagships” of thermophilic biocenoses which contribute a significant amount to the national species diversity in the depauperated Northern countries. *L. agilis*, a species which is very common in Southern Germany or Eastern Europe, was placed in the highest category of the European Fauna-Flora-Habitat directive because of its rarity in Britain and Sweden.

The main point to clarify is whether isolated populations are indigenous or introduced by humans. Genetic methods can provide useful data to solve this problem, but if the indigenous haplotype also occurs in the main range, they cannot provide a certain answer. For example in German *E. orbicularis*, the Eastern haplotype IIb is indigenous, whereas turtles from Western Germany -IIa – are found among many non-indigenous Southern European haplotypes (Fritz et al. 2004). Turtles with haplotype IIa could be introduced from France or Hungary, or could represent the last survivors of the indigenous population. The isolated West German Kaiserstuhl population of *L. viridis* (see Fig. 3) is now under suspicion of having been introduced, as *L. bilineata* would be the indigenous species expected there (Joger et al. 2001).

One main result of our genetic studies was the discovery of unique haplotypes at the Southern margin of most species ranges. Red lists and conservation plans in the Mediterranean and Caucasian countries must be directed to take special care of formerly ignored local endemics such as *E. trinacris*, *Z. lineatus*, and *H. viridiflavus xanthurus* in Sicily and Cantabria, *L. (agilis) boemica* in Armenia, Southern Spanish *N. maura* ssp. and Greek *N. tessellata* ssp.

Outside Europe, in Turkish Anatolia, Iran, and the Maghreb countries, increased research activities (particularly phylogeographic studies with molecular techniques) are needed to evaluate the high genetic diversity, identify relicts and cryptic species which are still unknown, and define conservation targets.

**Acknowledgments** We like to thank many colleagues – too numerous to mention individually – who provided tissues from different parts of Europe and non-European parts of distribution ranges. Robin Lawson helped with sequencing *Natrix* spp, Nik Stümpel and Alexander Zinenko sequenced additional *Vipera* samples and Toni Amann did initial work on the *Lacerta viridis* complex. Our work was financed with grants provided by the Deutsche Forschungsgemeinschaft (DFG Jo-134/7, Jo-134/9 and Wi-319/18).

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# Niche Conservatism among Allopatric Species of the Grasshopper Genus *Afrophaeoba* Jago, 1983 in the Eastern Arc Mountains (Tanzania)

Axel Hochkirch

**Abstract** The persistence of species is strongly determined by their ecological niches. It is therefore, of major importance to obtain knowledge on the niche attributes of relict species in order to conserve these species and understand their current distribution patterns. Niche conservatism has been proposed to explain distribution patterns of many taxa, particularly in the tropics. Indeed, the high gamma diversity in the tropics seems to be influenced by allopatric assemblages of shared genera, whereas species overlap is often relatively low. Here, the niche overlap of allopatric *Afrophaeoba* grasshopper species from the Eastern Arc Mountains (Tanzania) is studied and compared with niche overlaps among sympatric but distantly related species in the East Usambara Mountains. The results show that the genus *Afrophaeoba* exhibits a strong degree of niche conservatism. All congeneric species are confined to grassy forest edges. Niche overlap within this genus was generally higher than expected by chance and higher than among sympatric species, whereas the variance of niche overlaps between species pairs was low. While the sympatric species could be distinguished ecologically using a few microhabitat variables, a comprehensive data set for the *Afrophaeoba* species revealed only little resolution. The results support the hypothesis that diversification within tropical grasshoppers is not driven by niche divergence, but by allopatric isolation. Based on the niche quality of *Afrophaeoba*, it is proposed that the persistence of the genus (and probably other forest taxa) might also be possible in riverine forests. There is a strong need for studies on the potential role of gallery forests as corridors for gene flow among mountain forest taxa.

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## 1 Introduction

There is little doubt that the survival of populations is strongly influenced by their ecological niches (Thuiller et al. 2005). The occurrence of a relict species is, therefore, also determined by its niche quality, breadth and flexibility. Species or populations with a narrow and conserved niche are likely to be more prone to extinction than those with a broad niche or strong adaptability. Ecological niches of species are generally thought to be highly conserved (Peterson et al. 1999) and this seems to be particularly true for the tropics (Wiens and Graham 2005). Indeed, tropical mountain forests often maintain a species-rich fauna, and different mountain blocks have a low species overlap but a high number of shared genera (Hochkirch 1998). This pattern leads to high gamma-diversity (i.e. a high total biodiversity in larger tropical regions) with many relict species inhabiting single tropical mountains. The Eastern Arc (a mountain chain in East Africa) is an excellent area to study niche divergence of tropical relict species, as the mountains in this chain are covered by evergreen forests and separated by savannahs and dry woodlands (Lovett and Wasser 1993). The Eastern Arc belongs to the global hotspots of biodiversity (Myers et al. 2000) and is known for its great species richness with a high degree of endemism (Rodgers and Homewood 1982; Lovett and Wasser 1993; Burgess et al. 2007). This is particularly true for flightless insect species, such as most forest grasshoppers (Hochkirch 1998). One major reason for the high degree of endemism in the Eastern Arc fauna seems to be the comparatively stable climate during Pleistocene climatic fluctuations (Scharff 1993). Eastern Arc endemics are, therefore, thought to be relicts of formerly widespread forest taxa, which survived periods of drought at high elevations (Hochkirch 2001).

Studies on niche conservatism usually deal with climatic variables that might explain the geographic range of species (e.g. climate envelopes of congeneric species; Peterson et al. 1999). Other niche dimensions, such as the microhabitat preferences of tropical sibling species have rarely been considered. However, at a small spatial scale, sympatric insect species often partition niche space in terms of their microhabitat utilisation (Hochkirch 1995). Hence, it is necessary to compare the microhabitat requirements of allopatric species in order to address the question of niche conservatism. The flightless grasshopper genus *Afrophlaeoba* Jago 1983 consists of four allopatric species, each of which is endemic to a single mountain block of the Eastern Arc. Genetic and morphometric data suggest that the genus has been formerly more widespread in East Africa and that speciation took place after fragmentation of lowland and riverine forests during the early Pleistocene (Hochkirch 2001). The four species are morphologically rather similar but can be distinguished genetically and by multivariate morphometrics (Hochkirch 2001, 2005). Morphologically, the genus *Afrophlaeoba* represents the graminicolous type *sensu* Uvarov (1977). Most grasshopper species are specialised on a particular vegetation structure and many species exhibit behavioural adaptations to their microhabitat (Sänger 1977; Uvarov 1977). In many cases, there are also intersexual differences in microhabitat utilisation due to sex-specific differences in energy budgets (Hochkirch et al. 2007).

In the present paper, the microhabitat utilisation of all *Afrophlaeoba* species is studied to obtain information on the niche overlap among these allopatric species. Given that the hypothesis of niche conservatism is true, one would expect a high niche overlap among the four species. For comparison, the niche overlap of sympatric grasshopper species of the East Usambara Mountains is analysed. If niche overlap was greater among the sympatric (distantly related) species than among the allopatric (closely related) species, it would be likely that the specific vegetation structure of the mountain blocks has a stronger influence on niche overlap than the genetic relationship. The results might also help to reconstruct the ancestral niche of the species - information which is needed to draw conclusions on the former habitat connectivity.

## 2 Methods

### 2.1 Study Sites

The Eastern Arc Mountains are Pre-cambrian basement rocks, which have been faulted during the Miocene (Iversen 1991). The area consists of several mountain blocks with a high number of endemic species and genera (Burgess et al. 1998a). The more prominent rocks of the Arc are the Taita Hills (Kenya), North and South Pare Mts., East and West Usambara Mts., Nguu Mts., Nguru Mts., Ukaguru Mts., Rubeho Mts., Uluguru Mts., Malundwe Hill, Udzungwa Mts. and Mahenge Mts. (all in Tanzania). About 25% of the ca. 2,000 plant species and 66% of the 82 amphibian and reptile species occurring in the Eastern Arc are endemic (Lovett 1988; Howell 1993). The area is influenced by the Indian Ocean monsoon (Lovett 1993a) and the mountain blocks are separated by dry woodland and savannah. Marine drill-core data suggest that the coastal waters of East Africa were less influenced by Pleistocene climatic fluctuations (1–2°C) than other oceans (Prell et al. 1980). The stable temperatures of the Indian Ocean may have supported the high rainfall pattern of the Eastern Arc and the persistence of evergreen forests. The present climate of the Eastern Arc is mainly influenced by the seasonal movements of the intertropical convergence zone, which crosses the equator twice a year (Hamilton 1982). Subsequently, two rainy seasons occur in the East Usambara Mountains, while the rainy seasons merge to one long rainy season further South (e.g. in the Udzungwa Mts.). Secondly, the rainfall pattern is modified by oceanic currents and the positions of the mountains. The currents of the Indian Ocean (South Equatorial current) bring warm and humid waters to the East African coast, increasing the rainfall close to the coast and at the Eastern slopes of the mountains (Wasser and Lovett 1993). The Eastern Arc Mountains are known to receive the highest rainfall in East Africa (up to 2,000–3,000 mm per year). The temperatures are exceptionally low, with frosts occurring frequently above altitudes of ca. 2,000 m during July and August (Lovett 1998). The cool climate is mainly caused by cloudiness, which

decreases the quantities of incoming radiation (Kenworthy 1966). Parts of the Usambaras and Ulugurus have an almost per-humid climate (Lovett 1993a).

The vegetation zones of the Eastern Arc mainly follow the altitudinal and moisture gradients (Lovett 1993b). The floristic variation with the altitude is continuous without any abrupt changes (Hamilton 1998). Forests naturally extend from the lowlands of the Eastern slopes up to 2,400 m above sea level. However, at the lower slopes many forests have been cleared. At low altitudes the forest vegetation is rather similar to the lowland coastal forests, which occur fragmented along the Eastern coast of Africa (Burgess et al. 1998b). The lowlands between the Southern (and inland) parts of the mountain chain are mainly covered by miombo woodland (composed of the tree genera *Brachystegia*, *Isoberlinia* and *Julbernardia*) and receive an annual rainfall of 750–1,000 mm with a long dry season in between (Hamilton 1982).

## 2.2 Study Objects

The genus *Afrophlaeoba* consists of four flightless grasshopper species, each of which is endemic to a single mountain block (Jago 1983; Hochkirch 2001). *Afrophlaeoba usambarica* occurs in the East Usambara Mountains, *Afrophlaeoba euthynota* in the Uluguru Mountains, *Afrophlaeoba nguru* in the Nguru Mountains and *Afrophlaeoba longicornis* in the Rubeho and Ukaguru Mountains. The species have a continuous life cycle and adults can be found throughout the year (Jago 1983; Hochkirch 1996). They mainly inhabit grassy forest edges and clearings and seem to be graminivorous (Hochkirch 1996). All other species studied are endemics to the East Usambara Mountains or occur from this mountain block to the coast. They include distantly related taxa as well as a sister genus of *Afrophlaeoba* – *Parodontomelus arachniformis* – which is another graminicolous species that belongs to the *Afrophlaeoba* genus group. It occurs in the coastal forests of Tanzania, the East Usambara Mountains and in Zanzibar (Jago 1983). *Rhainopomma usambaricum* is distributed from the East Usambaras to the Shimba Hills in South-Eastern Kenya (Jago 1981). It occurs at forest edges, paths and clearings in dense and high foliage, where it mainly feeds on forbs (Hochkirch 1995). *Parepistaurus pygmaeus* is known from the East Usambaras and the Nguru Mountains (Green 1998), where it inhabits warm and sunny forest edges with low and dense vegetation (Hochkirch 1995). *Ixalidium transiens* is endemic to the East Usambara Mountains. It can be found among litter at the forest floor and feeds mainly on plant debris (Hochkirch 1995).

## 2.3 Data Collection

The practical fieldwork was performed from 06 December 1997 to 13 February 1998 (*Afrophlaeoba* and *Parodontomelus*). The data was recorded during a

short period of time to avoid seasonal influences. In order to collect data on the microhabitat preferences, the method described by Hochkirch (1995, 2001) was used. All data were obtained between 8:00 and 18:00 at the exact location of undisturbed individuals, which were haphazardly chosen. For each grasshopper the following information was recorded: date, time, study site, species, sex, behaviour, vegetation cover (divided into grasses, forbs, mosses, litter and bare ground, estimated in a circle of 25 cm radius around the individual), vegetation height (highest plant in a circle of 25 cm radius) and the substrate, on which the insect perched (including the categories grasses, forbs, bare ground, leaf litter, grass litter). Temperature and radiation was also recorded, but not included in the analyses as these values depend too much on weather conditions. In total, 300 records were available for the four *Afrophlaeoba* species and for *P. arachniformis*. For comparison, data of sympatric species of the East Usambara Mts. were included, which were recorded from 11 June 1994 to 13 November 1994 (*R. usambaricum*, *P. pygmaeus*, *I. transiens*). The data was obtained similarly to the above mentioned, but vegetation cover was not divided into different categories. In total, 732 records were obtained for *R. usambaricum*, 637 records for *P. pygmaeus* and 184 records for *I. transiens*.

## 2.4 Statistical Analysis

The Czechanowski index (Gotelli and Entsminger 2001) was applied to quantify niche overlap between the species for all microhabitat parameters. The Czechanowski index ranges from 0 (no shared resource states) to 1 (identical habit utilisation). To test whether the observed niche overlap differed from a random pattern we carried out null model analyses in EcoSim 7.0 (Gotelli and Entsminger 2001). EcoSim simulates patterns of niche overlap and compares these randomised results with the observed data matrix. We used the algorithm RA3 (Winemiller and Pianka 1990) to test for non-random niche overlap. In this procedure, the observed niche breadth of each species is retained, but the utilisation of any resource state is allowed, including categories that were available but not used by the species. For each data set 10,000 replicates were created in the simulation.

To test for correlations between vegetation parameters, multivariate ordination analyses were performed. Because of the long gradient length in the data set, a correspondence analysis (CCA) was performed as implemented in the community ecology package *vegan* 1.13-1 for R (Oksanen et al. 2005). To evaluate the most important variables for the species, the locations of the species were fitted onto the CCA plot, using the function “*envfit*” with 1,000 random permutations. This method also generates an  $R^2$  measure and significance values based on the probability that random permutations would yield a higher degree of fit than the true data (Oksanen et al. 2005). The multivariate analyses were carried out in R 2.7.1 (R Development Core Team 2008).

## 3 Results

### 3.1 Niche Overlap

The Ecosim analyses revealed that for all variables examined, niche overlap among *Afrophlaeoba* species was significantly greater than expected by chance, whereas for several variables the variance of the niche overlaps was smaller than expected (Table 1, Fig. 1). Averaged across all variables, niche overlap varied between 80.7 and 85.0% between congeneric species pairs. Compared with sympatric species from the East Usambara Mts., niche overlap was generally higher among *Afrophlaeoba* (Fig. 2). In the East Usambaras, niche overlap was also more variable (variance was greater than expected by chance), ranging from 28.0 to 85.4% (mean: 59.1%). The greatest niche overlap among the sympatric species was found between *A. usambarica* and *P. pygmaeus* which occur syntopically at forest edges in the East Usambaras, but have a different diet (Hochkirch 2001).

### 3.2 Multivariate Analysis

The first function of the correspondence analysis (CCA) for the vegetation parameters of the *Afrophlaeoba* data set (including *P. arachniformis*) explained 64.7% of the variance and the second function 13.1% (Fig. 3). The grasshopper species were significantly correlated with the vegetation matrix ( $P < 0.001$ ), but the resolution was relatively low ( $R^2 = 0.106$ ). The *Afrophlaeoba* species were plotted gradually along the first axis, while *P. arachniformis* was mainly found at patches with a great amount of leaf litter. For the complete data set (East Usambara grasshoppers combined with the *Afrophlaeoba* species), the CCA explained 68.6% of the variance and the second axis 31.4% (Fig. 4). Again, the species were significantly correlated with the vegetation data ( $P < 0.001$ ), but with a greater resolution ( $R^2 = 0.276$ ). In the ordination plot (Fig. 4), the distance between the sympatric species was greater than between the *Afrophlaeoba* species.

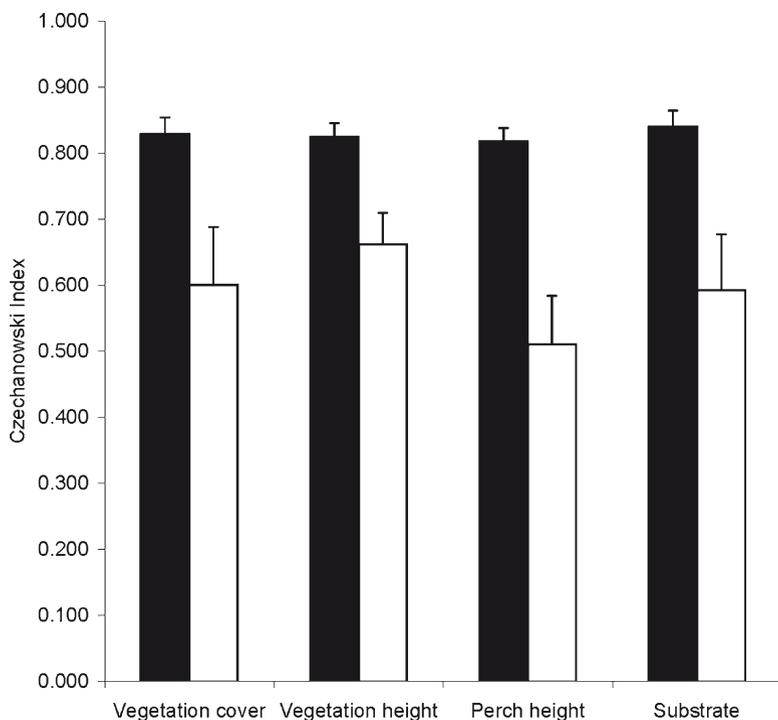
## 4 Discussion

The results support the niche conservatism hypothesis. Compared to the marked niche differences among sympatric grasshopper species of the East Usambara Mts., niche overlap was high among the four allopatric, congeneric species. For all microhabitat variables analysed, niche overlap was significantly greater than expected by chance. A strong niche overlap was also reported for other traits such as phenology, diet and microclimatic factors (Hochkirch 2001). The results support equilibrium models of biodiversity, suggesting that niche specialisation plays a major role in the

**Table 1** Czechanowski indices for each species pair of *Afroplitaoba* and results of a null model analysis\*

Czechanowski index	Vegetation cover	Grass cover	Forb cover	Leaf litter	Bare ground	Vegetation height	Perch height	Substrate
<i>A. euthynota-A. longicornis</i>	0.893	0.687	0.757	0.763	0.917	0.880	0.799	0.863
<i>A. euthynota-A. nguru</i>	0.807	0.860	0.817	0.617	0.907	0.863	0.800	0.774
<i>A. euthynota-A. usambarica</i>	0.840	0.913	0.913	0.760	0.977	0.817	0.888	0.888
<i>A. longicornis-A. nguru</i>	0.737	0.723	0.753	0.687	0.983	0.810	0.872	0.911
<i>A. longicornis-A. usambarica</i>	0.793	0.697	0.717	0.893	0.910	0.840	0.768	0.837
<i>A. nguru-A. usambarica</i>	0.900	0.870	0.850	0.783	0.900	0.733	0.779	0.764
Observed mean	0.828	0.792	0.801	0.751	0.932	0.824	0.818	0.840
Simulated mean	0.265	0.664	0.545	0.303	0.297	0.384	0.448	0.361
P (obs > exp)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
observed variance	0.0003	0.0101	0.0053	0.0087	0.001	0.0027	0.0025	0.0036
simulated variance	0.0260	0.0064	0.0085	0.0263	0.1296	0.0107	0.0120	0.0466
P (obs < exp)	0.031	0.864	0.298	0.051	0.013	0.046	0.029	0.013

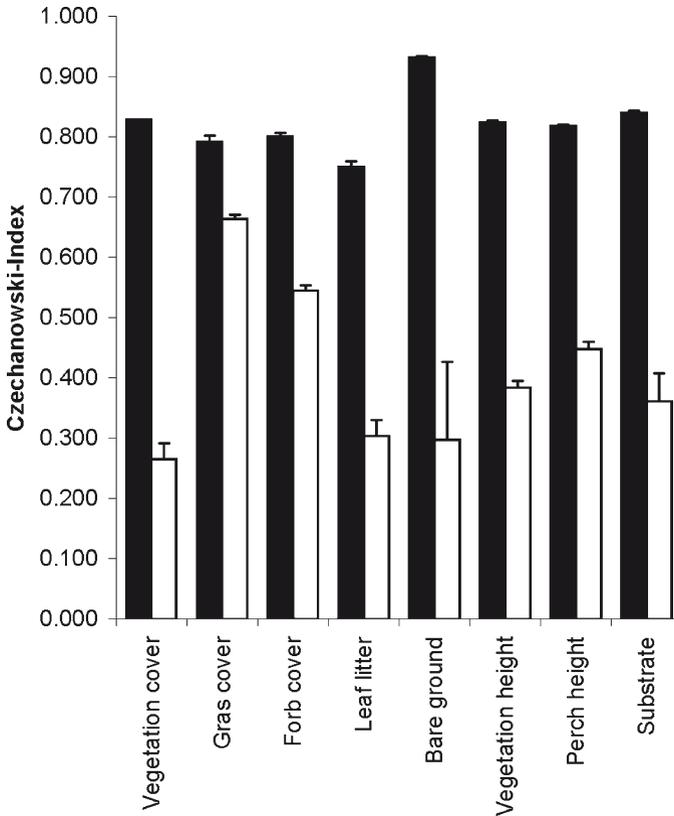
\*The analyses were performed in Ecosim 7.0 Gotelli and Entsminger (2001). For each data set 10,000 replicates were created in the simulation



**Fig. 1** Mean observed niche overlaps (*black*) and mean simulated niche overlaps (*white*) among allopatric *Afrophaeoba* species. Error bars are variances. All observed niche overlaps were significantly higher than expected by chance

generation of the high alpha biodiversity in the evergreen mountain forests (Linsenmair 1990). Indeed, the five species from the East Usambara Mts. can be clearly distinguished ecologically: *I. transiens* occurs in the ground litter of the forest, where it feeds on litter; *R. usambaricum* occurs in the dense and high vegetation of small forest gaps, feeding on forbs; *P. arachniformis* occurs in the sparse and low grassy vegetation of forest gaps, feeding on grasses; *P. pygmaeus* and *A. usambarica* occur syntopic in the dense and low vegetation of the forest edge (which is also illustrated by a strong niche overlap), but *P. pygmaeus* is a forb feeder, whereas *A. usambarica* is graminivorous. Although the niche overlap between *P. arachniformis* and *A. usambarica* is also relatively high (78.8%), these two species never co-occur in the East Usambaras as *P. arachniformis* is confined to the forest interior, while *A. usambarica* is found at the forest edge (Hochkirch 1996, 2001).

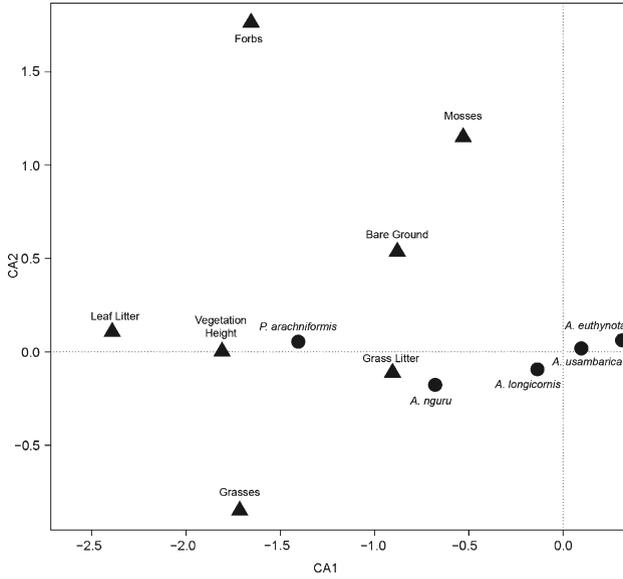
The Eastern Arc is believed to have served as a major refuge area during periods of drought. This hypothesis is supported not only by a high number of endemic species, but also by phylogenetic studies (Hochkirch 2001). Some taxa have their closest relatives in West Africa (e.g. the flightless grasshopper genus *Loveridgacris* Rehn, 1954), some in Madagascar (e.g. the groundhopper genus *Cryptotettix*



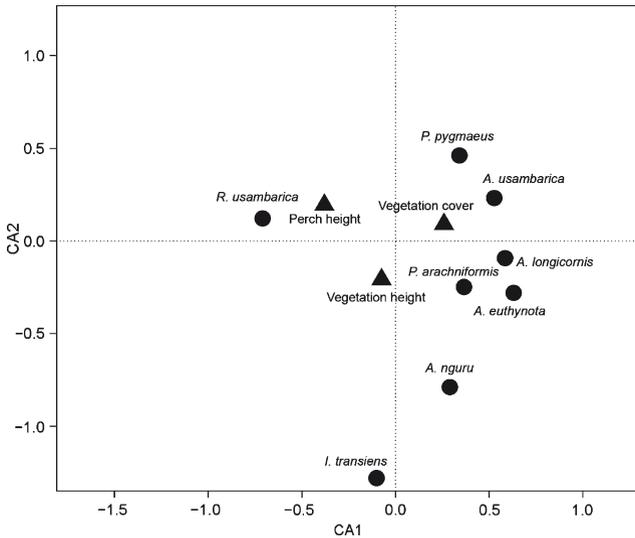
**Fig. 2** Mean observed niche overlap (Czechanowski Index) among allopatric *Afrophlaeoba* species (*black*) and sympatric grasshopper species in the East Usambara Mts. (*white*). Error bars are standard errors. Missing error bars are too small to be displayed

Hancock, 1900) and some even in Southeast Asia (e.g. the Udzungwa forest partridge *Xenoperdix udzungwensis* Dinesen, 1994). Climatic stability is of major importance for the survival of such relicts (Lovett and Friis 1996), but interestingly the majority of neoendemics are located in the same areas as relict species (Fjeldså and Lovett 1997). The low number of endemic mammals in the Eastern Arc (Stanley et al. 1998) suggests that the forest refuges in East Africa were rather small.

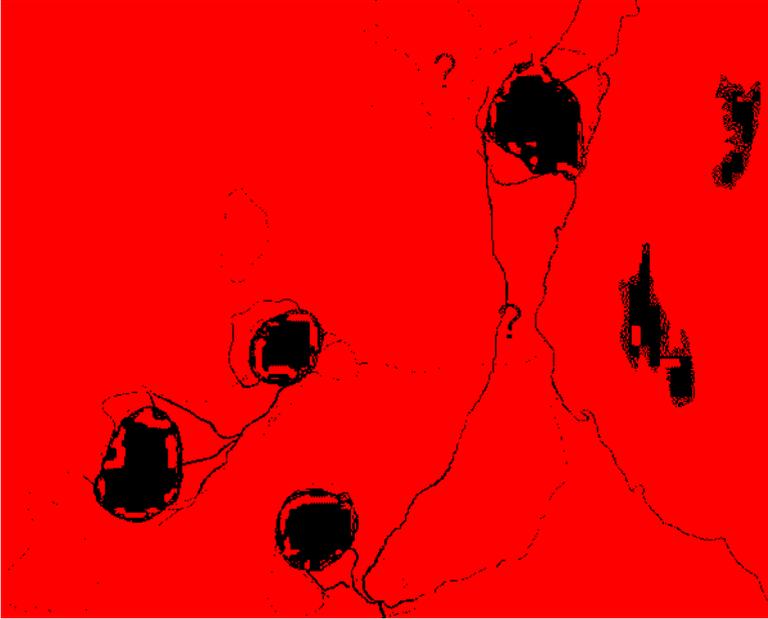
The genus *Afrophlaeoba* represents a relatively young radiation in the Eastern Arc. Molecular phylogenetic analyses suggest that the oldest split within the genus originated during a period of drought during the early Pleistocene (Hochkirch 2001). Two vegetation types are thought to have provided corridors for gene flow among the formerly widespread populations of these flightless insects: coastal forests and riverine forests (Hochkirch 2005). A scenario of the evolutionary divergence of the genus (Hochkirch 2001) suggests that the ancestral distribution comprised at least the formerly widespread coastal forests, adjacent riverine forests



**Fig. 3** Plot of the first two axes of a correspondence analysis of the microhabitat data of *Afrophlaeoba*. The first axis explains 64.7% of the variance; the second axis explains 13.1% of the variance



**Fig. 4** Plot of the first two axes of a correspondence analysis of the microhabitat data of all grasshopper species examined. The first axis explains 68.6% of the variance and the second axis explains 31.4% of the variance. Sympatric species are represented by black dots; the three allopatric species (*A. nguru*, *A. longicornis*, *A. euthynota*) are shown in grey



**Fig. 5** Vicariance scenario for the fragmentation of populations of *Afrophlaeoba* (question marks represent unconfirmed recent records, lighter shadings represent higher ages; the off-shore islands of Zanzibar and Pemba are shown in black; modified from Hochkirch (2005))

and parts of the Eastern Arc (Figs. 5). The Wami River basin might have acted as corridor for the mountain and coastal populations. The phylogenetic relationships suggest an initial fragmentation of the “Wami River Connection” close to the coast, probably in the Zanzibar rain shadow which is the driest area at the coast (Hawthorne 1993). The breakdown of this habitat connection was followed by fragmentation of the coastal forests shortly after that. It is well known that coastal forests were once more widespread in areas that are now woodland (Clarke and Karoma 2000). Interestingly, the Zanzibar rain shadow represents an area where a number of other taxa show North-South disjunctions (Hawthorne 1993; Robbrecht 1996; Tattersfield 1998; Lindqvist and Albert 1999). The “Wami River Connection” between the Nguru Mountains and the Rubeho Mountains must have remained stable for a longer time and might even still exist.

Ecologically, the *Afrophlaeoba* species are not fully restricted to dense evergreen forests as suggested by Jago (1983); rather, they occur at forest edges and are able to persist in plantations of mango trees or in gallery forests, which might have served as corridors for gene flow between the mountain populations (Hochkirch 2005). It has also been assumed for other taxa with a disjunct distribution in the East African Mountains, that these might have once occurred continuously in lowland and riverine forests (Hamilton 1982; Brandl et al. 1996). Indeed, several taxa are known to be shared by the Coastal Forests, the Eastern Arc and several gallery

forests, such as the Pongwe and Wami River valleys (Hoffman 1993; Kellman et al. 1994; Clarke 2000). These riverine forests might also have served as refuges for forest taxa in periods of drought, since they are less dependent on rainfall but more on groundwater (Medley 1998). Even small remnants of gallery forests might have been large enough to maintain populations of arthropods (Scharff 1992). There is a strong need for research on the possible interconnections between mountain forest populations, which might be provided by riverine forests.

**Acknowledgements** I thank my supervisor, Prof. Dr. Dietrich Mossakowski (Bremen, Germany), and my local contact, Prof. Kim Howell (Dar es Salaam, Tanzania), for their help and advice. This work was financially supported by the “Stifterverband für die Deutsche Wissenschaft” and by a “DAAD Doktorandenstipendium im Rahmen des gemeinsamen Hochschulonderprogramms III von Bund und Ländern”. The Commission for Science and Technology (COSTECH, Dar es Salaam, Tanzania) provided the research clearance for this study.

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# Conservation Genetics and Phylogeography of the Threatened Corsican and Barbary Red Deer (*Cervus elaphus corsicanus* and *C. e. barbarus*)

Frank E. Zachos, Ghaiet M. Hajji, and San S. Hmwe

**Abstract** The two red deer subspecies *C. e. corsicanus* (Corsican red deer, endemic to the Tyrrhenian islands of Sardinia and Corsica) and *C. e. barbarus* (Barbary red deer, presently confined to a small area along the Tunisian-Algerian border) are classified by the IUCN as “Endangered” and “Lower risk (near threatened)”, respectively (until recently, the Barbary red deer’s status was “Vulnerable”). Both subspecies underwent severe bottlenecks in the twentieth century. We review our results on genetic variability and differentiation in these two subspecies at polymorphic microsatellite loci and sequences of the mitochondrial control region and discuss their bearing on conservation and phylogeography. Our analyses yielded very low genetic variability for *C. e. corsicanus*. The Barbary red deer also showed low variability values in a European comparison, but diversity was not as low as might have been feared in the light of the population’s history. Analyses of molecular variance, assignment tests and factorial correspondence analysis did not yield any signs of differentiation among the Tunisian subgroups studied, whereas there was clear evidence of a differentiation between the Sardinian and the Corsican red deer population. The phylogeographic analysis showed close relationships between *C. e. corsicanus* and autochthonous Italian deer at two unlinked sets of nuclear loci, suggesting as the most likely scenario an introduction of Italian mainland red deer to the Tyrrhenian islands, from where animals were probably translocated to North-Africa.

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## 1 Introduction

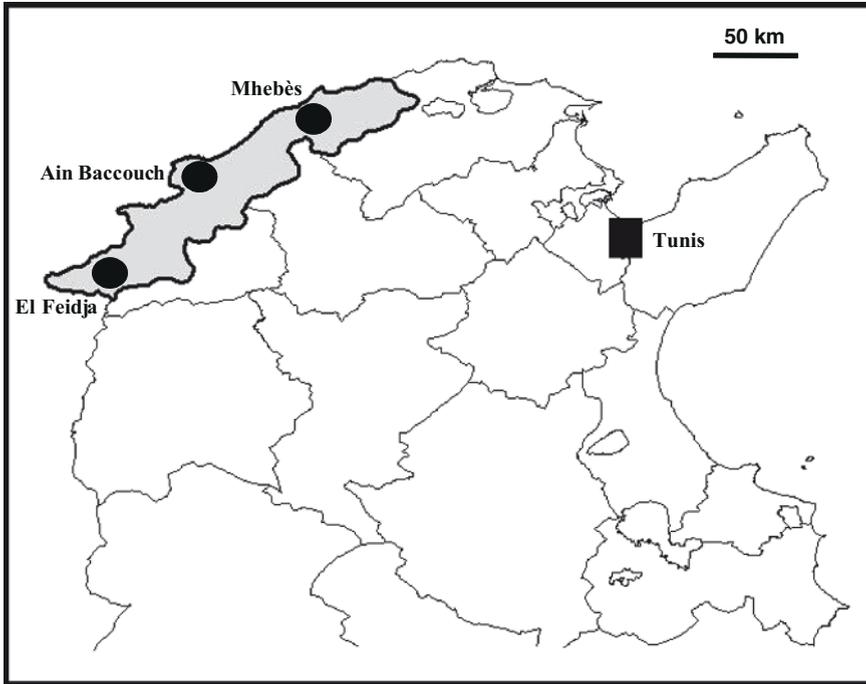
The red deer (*Cervus elaphus*) is not an endangered species as a whole. Two genetically and morphologically distinct Western-Palearctic populations, however, are listed by the World Conservation Union as “Endangered” (*C. e. corsicanus*, the Tyrrhenian or Corsican red deer) and “Lower Risk” (near threatened) (*C. e. barbarus*, the North-African Barbary red deer). In the present review, we shall give a summary on our work on these two subspecies with regard to (1) genetic variability and differentiation of subpopulations and (2) the phylogeographic origin of the Tyrrhenian red deer and its relationship to the Barbary red deer.

## 2 Conservation Genetics

### 2.1 Barbary Red Deer

Apart from recent introductions, the Barbary red deer (*C. e. barbarus* Bennett, 1833) is the only African deer taxon. The historical range of this deer covered large parts of Algeria, Tunisia and possibly Morocco, but at present the Barbary deer is restricted to a small area of cork oak and pine forest on the Tunisian-Algerian border (Fig. 1) – a concentration which makes it particularly susceptible to diseases and forest fires, which are not uncommon in the region (Kock and Schomber 1961; Cowan and Holloway 1973; Dolan 1988; Oumani 2006). The Barbary red deer is listed in Appendix III by CITES (a recent proposal to list it in Appendix I was rejected in June 2007), and its IUCN status has recently been changed from “Vulnerable” to “Lower Risk (near threatened)” (Wemmer 1998; IUCN Red List 2007). Barbary red deer are characterised by a simplified antler structure (frequent lack of the bez tine) and by white spots running in longitudinal lines (Dolan 1988; Geist 1998). They suffered a severe bottleneck with only seven specimens left in Tunisia in 1962, two of them in Ain Baccouch and five in Ain Seraouia (Van Dijk 1972), which, however, were augmented by animals from Algeria crossing the border (Dolan 1988; Wemmer 1998). Consequently, hunting was banned, and in 1966, a reserve of 417 hectares with 17 individuals was established at El Feidja, about 190 km Southwest of Tunis, from where the deer can emigrate freely (Direction Générale des Forêts – hereafter abbreviated as DGF – 1994). This reserve is located within the El Feidja National Park, which also includes an additional enclosure for the Barbary deer. Subsequently, an area of ca. 200 km along the Mediterranean in Northern Tunisia was colonised (Meyer 1972; Dolan 1988).

Apart from Algerian immigrants, the El Feidja population is the origin of all Barbary deer present in Tunisia. Between 1978 and 1996, several reintroductions of this species into different reserves, mainly Ain Baccouch (Tabarka) and Mhebès (Séjnane), were carried out (DGF 1994). Population size at El Feidja seems to have been about 100 individuals since 1972 (Van Dijk 1972; Dhoub 1998; Oumani 2006).



**Fig. 1** Distribution of the Barbary red deer in Tunisia (*shaded area*) and location of sample sites. Figure taken from Hajji et al. (2007)

For Mhebès, Oumani recorded 80 individuals in 2002 and a similar number (70) in 2006 (Oumani 2006). The present number of deer at Mhebès is rather low compared with the number of founders (39 individuals) in 1979 (DGF 1994). The fact that neither of the two reserve populations (El Feidja and Mhebès) has increased is explained by Oumani (2006) with the dispersal of the deer from the reserve areas into adjacent habitats. Sadly, poaching is still also an important factor in limiting the population growth of the Barbary deer in Tunisia.

The total population size of *C. e. barbarus* in Tunisia was estimated by Oumani (pers. comm. 2006) to be about 800 individuals. This is in clear contrast to the census results obtained by the DGF according to which there are 2,000 deer in Tunisia (DGF 1994; see also Dolan 1988 who gives the same number). In addition to protection through government programmes, the WWF, in the context of the Regional Euro-Mediterranean Programme for the Environment (SMAP), is coordinating a project at El Feidja and Mhebès including studies on the population size and ecology of the deer in Northwestern Tunisia and the establishment of a deer enclosure at Mhebès. These conservation measures, however, are still sometimes thwarted by the government as was shown by the sale of 10 red deer from an enclosure in El Feidja in 2007 to a prince who wanted them for his private zoo in Morocco. In addition to these 10 animals, many more panicked during the capturing, jumped over the enclosure's fence and have not returned since.

Apart from the inclusion of some African samples in phylogenetic or phylogeographic studies (Polziehn and Strobeck 2002; Ludt et al. 2004; Hajji et al. 2008) there has only been one genetic study of Barbary red deer. Hajji et al. (2007) analysed 30 Tunisian Barbary red deer from El Feidja ( $n=12$ ), Ain Baccouch ( $n=8$ ) and Mhebès ( $n=10$ ). Samples were collected in the field (faeces) or came from skin and hair of captured or accidentally killed or injured deer.

A stretch of 680 bp of the mitochondrial control region was successfully amplified and sequenced in 20 individuals. Amplification of 13 polymorphic microsatellites was successful in 19 red deer, a subset of 8 loci was amplified in all 30 individuals. Two distinct haplotypes were found differing by 16 transitions and being distributed evenly among the 20 specimens, occurring 11 and 9 times, respectively. This resulted in a low or intermediate haplotype diversity but in a rather high nucleotide diversity (Table 1), which is typical of populations having undergone a transient bottleneck where many haplotypes from the polymorphic original gene pool are lost through drift and those remaining are usually not closely related to one another (Nei et al. 1975).

The microsatellite loci analysed did not show any significant signs of linkage disequilibrium. Neither did tests for distortion of our results through stutter bands, large allele dropout or null alleles. Observed and expected heterozygosity values were 0.46 and 0.78, respectively, allelic diversity was 5.60 (Table 1).

A statistical test for a signature of the bottleneck in the microsatellite data (based on heterozygosity values relative to those expected under mutation-drift equilibrium and implemented in the Bottleneck software by Cornuet and Luikart 1996) was highly significant ( $p=0.00018$ ).

We particularly tested our three sample sites El Feidja, Ain Baccouch and Mhebès for signs of (incipient) differentiation by calculating overall and pairwise  $F_{ST}$  and  $R_{ST}$  values (the latter being a microsatellite-specific analogue of  $F_{ST}$ ) and by carrying out assignment tests in which individuals are assigned to the population of which their genotype is most typical. Additionally, a factorial correspondence analysis (FCA) was also conducted to see if this approach would yield any clusters of individuals related to their geographical origin. Positive signs of differentiation were not found in any of these calculations in that none of the  $F_{ST}$  or  $R_{ST}$  values were significantly different from zero; the assignment tests showed very high error rates (about 60%, which is not much lower than expected under random assignment to three populations); and there were no clusters of individuals in the FCA (not shown).

In Ain Baccouch, only one of the two haplotypes was found, but this is probably an artefact of the small sample size (control region amplification was successful in seven animals from Ain Baccouch) and may not be interpreted as evidence of differentiation. Consequently, the Tunisian red deer can still be considered a single, genetically homogeneous population.

The genetic data clearly reflect the population's bottleneck and show low genetic diversity for both mitochondrial control region sequences and nuclear microsatellite loci. However, compared to other populations known to be bottlenecked and/or inbred (e.g., Hasselbusch, Mesola or Corsica/Sardinia), variability values were not

**Table 1** Genetic variability at microsatellite loci and the mitochondrial control region in the Tunisian and European red deer populations

Population	H <sub>O</sub>	H <sub>E</sub>	AD	HD	π [%]
Tunisia	0.46	0.78	5.6	0.529	1.266
Sardinia	0.48	0.66	–	0.872	0.473
Corsica	0.54	0.64	–	–	–
Mesola	0.51	0.62	–	0	0
Hasselbusch	0.58	0.66	4.9	0.493	0.337
Val di Susa	0.75	0.85	8.0	0.591	0.733
Tarvis	0.76	0.81	–	0.750	0.650
Southern Spain	0.65	0.80	–	0.652	1.318
Bulgaria	0.74	0.85	–	0.667	0.986
Romania	0.54	0.88	–	0.500	0.644
Spain	0.66	0.78	7.5	–	–
Scotland	0.44	0.81	–	0.478	0.536

H<sub>O</sub> and H<sub>E</sub>: observed and expected heterozygosity, AD allelic diversity (only given when sample size was comparable, i.e. between 16 and 22), HD haplotype diversity, π: nucleotide diversity. Mesola, Val di Susa and Tarvis are on the Italian mainland. The values for Corsica are from Hajji et al. 2008. Hasselbusch is a strongly inbred and isolated population in Northern Germany (see Zachos et al. 2007 for details). The data for Sardinia and Mesola are from Hmwe et al. (2006a), those for Val di Susa, Tarvis, Southern Spain and Bulgaria from Zachos et al. (2003), and the Spanish, Romanian and Scottish values are taken from Martinez et al. (2002), Feulner et al. (2004) and Hmwe et al. (2006b). For details of calculations see Hajji et al. (2007)

as low as might have been expected in the light of the population's demographic history (Table 1, Hmwe et al. 2006a). This may be due partly to immigration of Algerian animals. The Algerian population is estimated to have been about 300–400 head in the early 1950s before the Algerian war for independence and 400–600 in the mid-1970s (Salez 1959; Kock and Schomber 1961; Wemmer 1998; for an estimate on population density in Algeria cf. Burtthey et al. 1992). The total Algerian population was estimated to be approximately 2,000 in the late 1980s (Dolan 1988) but since the official number for Tunisia is dubious, the same might also hold for Algeria.

Conservation measures should concentrate on the reduction of poaching, which still plays a major role in the mortality rates of Barbary red deer. At the moment, efforts are being made by the WWF to strengthen anti-poaching legislation. In addition, the distribution range of the Barbary red deer should be further extended to reduce its susceptibility to environmental stochasticity such as forest fires and diseases.

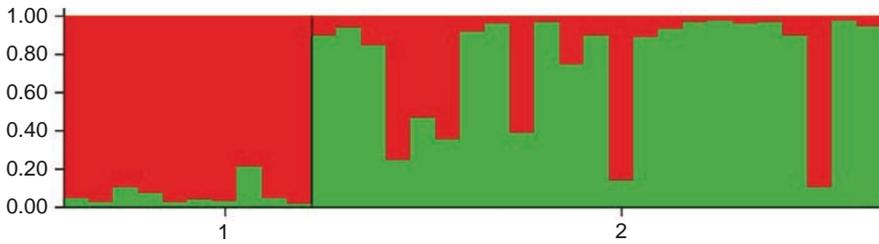
The analysis of Hajji et al. (2007) and ongoing field studies in Tunisia have yielded valuable data on *C. e. barbarus* but information about these deer outside Tunisia is much needed. There are still rumours of Barbary red deer having survived in Morocco. This sounds unlikely but should be investigated nonetheless. The most important future steps, however, must concentrate on Algeria. The population size in Algeria and the number of migrants between Algeria and Tunisia need to be known. An assessment of the genetic variability in Algerian red deer and

whether they are differentiated from the Tunisian population or not are pre-requisites of an effective bilateral management which must be the ultimate goal in the conservation of these last African red deer. Fortunately, plans for such bilateral protection measures are under way at present.

## 2.2 *Tyrrhenian or Corsican Red Deer*

The Corsican red deer (*C. e. corsicanus* Erxleben, 1777) is the smallest of the existing red deer (Dolan 1988). It is confined to the Tyrrhenian islands Corsica and Sardinia and classified as “Endangered” by the IUCN (Wemmer 1998; IUCN Red List 2007). In the second half of the twentieth century, it underwent a severe bottleneck with only 100–200 animals remaining (Krumbiegel 1982; Dolan 1988; Kidjo et al. 2007 and references therein), but has recovered well since so that recently it has been suggested that the Tyrrhenian red deer be downgraded to “near threatened” in the IUCN Red List (Kidjo et al. 2007). Present numbers are given as at least 2,700 head by Lovari et al. (2007), but in line with Banwell (1998), who gives an estimate of only 1,500 specimens, another recent figure is “slightly >1,000” (Kidjo et al. 2007). After its extinction around 1970, the Corsican population was re-founded with altogether 14 Sardinian animals (of which one male died shortly after its introduction) between 1985 and 1994 (Dolan 1988; Gonzalez and Kidjo 2002; Kidjo et al. 2007). The introduced deer were at first kept in enclosures, but since 1998 there have been repeated releases into the wild so that in 2004, there were about 200 free-ranging animals on Corsica and another 50 in enclosures (see Kidjo et al. 2007 who give a detailed report on the extirpation and reintroduction history of the red deer on Corsica).

The first assessment of genetic variability based on five microsatellite loci and 332 bp of the mitochondrial control region yielded very low variability values for 17 Sardinian red deer in a European comparison (Zachos et al. 2003). A more recent study, based on a larger sample size of 29 Sardinian red deer and an extended panel of 12 microsatellite loci as well as a larger fraction of the mitochondrial control region (531 bp) confirmed these results (Hmwe et al. 2006a, see values in Table 1). Microsatellite variability was nearly as low as for Mesola, a relict population of truly autochthonous Italian mainland red deer which mark the lower end of genetic diversity in European-type red deer. The comparatively high number of eight haplotypes found (defined by seven polymorphic sites) may be a consequence of more than one colonisation of or introduction to the island. Only a few females would be enough to have caused this mitochondrial pattern without significantly increasing nuclear genetic variability. We have recently analysed a panel of eight further microsatellite loci, which show the same picture with respect to variability (Hajji et al. 2008), but also included 10 red deer from Corsica in our analysis. Genetic variability of the Corsican animals was still a little lower than that for Sardinia (observed and expected heterozygosity and allelic richness were 0.54, 0.64 and 3.50 for Corsica and 0.53, 0.71 and 4.40 for Sardinia), but the differences were



**Fig. 2** Bayesian structure analysis of Corsican (1) and Sardinian (2) red deer with the STRUCTURE software (Pritchard et al. 2000). All Corsican animals were assigned to one cluster (*dark grey*) with high probabilities, whereas all but six Sardinian deer were assigned to a second cluster (*light grey*) with similarly high probabilities. When the data were tested for  $K=3$  clusters (which yielded a higher overall probability but is not shown), only two of these six Sardinian specimens were grouped with the Corsican red deer, while the other 21 Sardinian red deer were assigned to two “non-Corsican” clusters

not significant. Interestingly, however, signs of considerable differentiation were found between the Corsican red deer and their population of origin on Sardinia. Pairwise  $F_{ST}$  for these two populations was about 15% and significantly different from zero, and the number of loci not in Hardy-Weinberg-Equilibrium due to an excess of homozygotes increased considerably (from two and zero in the single populations to five in the pooled data set), which is indicative of a Wahlund effect, i.e. a reduction in homozygosity relative to Hardy-Weinberg expectations due to substructuring of the total population. Further, assignment tests assigned more than 90% of the Corsican and Sardinian animals to their correct population of origin, and a Bayesian structure analysis without a priori assumptions of geographical origin also yielded distinct clusters roughly equivalent to the two islands (Fig. 2). Finally, a tree based on genetic distances among all individual red deer from Corsica and Sardinia also showed a non-random pattern in that one of the two main branches comprised exclusively Sardinian animals while the other comprised pre-dominantly Corsican deer (not shown).

### 3 Phylogeography of the Corsican Red Deer

The origin of the Corsican red deer has been a matter of considerable debate (reviewed in Vigne 1988 and Zachos and Hartl 2006). The two main questions are: (1) where did it come from and (2) how did it reach the Tyrrhenian islands – naturally during a glacial regression of the sea-level or through human introduction? The latter hypothesis was already put forward at the end of the nineteenth century (Lydekker 1898), and Groves (pers. comm. to Geist 1998) thought that the Tyrrhenian red deer were introduced from North-Africa by the Phoenicians. It has recently been shown that hares (*Lepus* sp.) were introduced to Sardinia from Africa (Scandura et al. 2007), so this might also hold for red deer. In line with this,

Ludt et al. (2004) and Skog et al. (2009) found close relationships between Sardinian and Barbary red deer based on sequences of mitochondrial DNA (cytochrome b and control region; see also Sommer et al. 2008 for a review on red deer phylogeography). Flerov (1952) regarded the Corsican red deer to be closely related to the Barbary red deer and the Spanish subspecies *C. e. hispanicus*, and Zachos et al. (2003) and Hmwe et al. (2006a) found close similarities at the mitochondrial control region between Sardinian and Spanish red deer. This was, however, not corroborated in more comprehensive studies (Ludt et al. 2004; Skog et al. 2009). An early genetic hypothesis based on mitochondrial RFLP markers linked *C. e. corsicanus* with Bulgaria and the Near East (Hartl et al. 1995) but none of the subsequent analyses yielded further evidence in favour of this assumption.

The only other genetically substantiated hypothesis as to the origin of the Tyrrhenian red deer is based on nuclear microsatellite loci. Zachos et al. (2003) and Hmwe et al. (2006a) found close relationships between Sardinian and European mainland red deer, in particular those from the Mesola wood in the Po delta (the relationship of mitochondrial control region sequences derived from Sardinian and Mesola red deer was ambiguous). The Mesola population is important from a phylogeographic point of view as it is the only surviving, truly autochthonous red deer population in the Italian peninsula, thus very probably at least partially representing the original Italian gene pool (Mattioli 1990). This hypothesis matches with morphological analyses which showed close similarities between Holocene red deer from Southern Italy and samples from the extinct original Corsican red deer population (Vigne 1988). According to this hypothesis red deer either naturally colonised the Tyrrhenian islands from the Italian mainland during a glacial period in the Pleistocene when the sea-level was considerably lower (for details see Hmwe et al. 2006a) or were introduced to Sardinia and Corsica by man. Putative Pleistocene red deer fossils are known from Corsica (*C. e. rossii*, Pereira 2001), but the extant Tyrrhenian red deer can hardly be considered a descendant of these early deer because, in spite of a very well-analysed fossil record, no red deer were found between the early Holocene (ca. 11500 cal. BP) and the mid-Holocene (5000 cal. BP or later) (Vigne et al. 1997; Vigne 1999; Costa et al. 2003). Sites from the Late Neolithic (4500 cal. BP) in Sardinia and from Classical Antiquity in Corsica, however, have frequently yielded red deer bones (Fonzo 1987; Vigne 1988). This is clearly indicative of human introduction after the indigenous deer populations had become extinct (Vigne 1992, 1999; Schüle 1993; Masetti 1998, 2002a). The Corsican population, possibly much younger than the Sardinian one, could have been founded with introduced animals from Sardinia or directly from the Italian mainland. Such introduction events were not uncommon at the time, and deer are known to have been introduced to many islands as early as the eleventh millennium BP (Vigne 1993; Masetti 1996, 2002a,b).

To shed further light on this issue, red deer from Corsica and Sardinia together with a selective sample of numerous European-type red deer were analysed for the first time, among them both Mesola and North-Africa (Hajji et al. 2008; see Fig. 3).

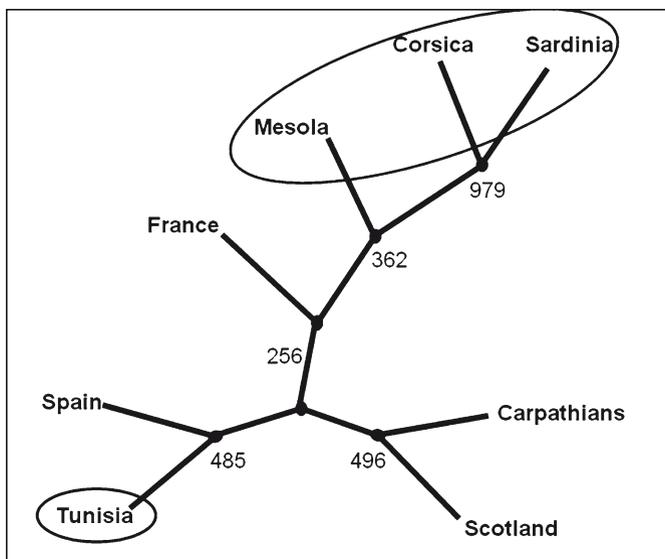
The study, based on eight nuclear microsatellite loci, yielded close relationships of *C. e. corsicanus* to red deer from both Mesola and North-Africa, as reflected, for



**Fig. 3** Geographical location of sample sites studied in a phylogeographic analysis by Hajji et al. (2008). 1: Scotland (*C. e. scoticus*,  $n=9$ ), 2: France (*C. e. hippelaphus*,  $n=14$ ), 3: Southern Spain (*C. e. hispanicus*,  $n=21$ ), 4: Tunisia (*C. e. barbarus*,  $n=30$ ), 5: Sardinia (*C. e. corsicanus*,  $n=23$ ), 6: Corsica (*C. e. corsicanus*,  $n=10$ ), 7: Mesola (*C. e. hippelaphus*,  $n=25$ ), 8: Romanian Carpathians (*C. e. montanus*,  $n=17$ ). The arrow denotes the most probable direction of introductions

example, by the fact that more than 50% of all alleles found in Mesola and Tunisia, respectively, were alleles also recorded in the Tyrrhenian red deer. However, the analyses favoured Mesola as the closest relative of the Tyrrhenian red deer. This was evident from allele frequencies and distributions at single loci, from different parameters of genetic distance and from the resulting trees (see Fig. 4). Nei's standard distances (Nei 1972), chord distances (Cavalli-Sforza and Edwards 1967) and proportion-of-shared-alleles distances (Bowcock et al. 1994, calculated as 1-Dps) between *C. e. corsicanus* and Mesola were 0.745, 0.117 and 0.585, while the respective values between *C. e. corsicanus* and Tunisia were 1.015, 0.126 and 0.640. Assignment tests did not yield misassignments of *C. e. corsicanus* specimens except for the individuals incorrectly assigned between Sardinia and Corsica (see above).

Rather unexpectedly, the study also yielded a quite close genetic proximity between *C. e. corsicanus* and the French red deer population. This may be due to introductions from France to the Tyrrhenian islands (which cannot be totally ruled out, although there are no records confirming this) or, more probably, due to the limited number of loci (eight) used in the analyses and/or length homoplasy.



**Fig. 4** Neighbour-joining tree based on pairwise chord distances derived from allele frequencies at eight nuclear microsatellite loci. Numbers at nodes refer to support from 1,000 bootstrap replicates. Figure modified from Hajji et al. (2008)

Perhaps the emerging picture would have been clearer had the animals been genotyped for more loci. This would also explain the rather low bootstrap support for nodes in our trees. Null alleles, stutter bands and allelic dropout, potential error sources known to be able to distort analyses based on microsatellites, were tested for with the MICRO-CHECKER software (van Oosterhout et al. 2004) and could be shown not to have influenced the data (see Hajji et al. 2008 for details).

In general, however, the results are in accordance with a priori expectations. They confirm those of Hmwe et al. (2006a) in that Mesola turned out to be the probably closest extant relative of the Tyrrhenian red deer. The recent study (Hajji et al. 2008) was based on eight completely different and unlinked nuclear loci, which further adds to the credibility of the main conclusion.

Nevertheless, although not as unequivocal as with mitochondrial sequence data, nuclear genetic analyses also confirmed a close relationship of Tyrrhenian and Barbary red deer. It is still unclear if there are true *Cervus* fossils in the Late Pleistocene of North-Africa (Cheylan 1991), and it is considered more probable that the modern Barbary red deer go back to introductions by humans in Holocene times (Kowalski and RzebiK-Kowalska 1991), although Hamilton (1978) thought that the Barbary red deer reached Africa via Sicily. Putting together both the palaeontological and the genetic results, it seems very likely that the Tyrrhenian red deer were introduced to Corsica and Sardinia from mainland Italy and that these deer were subsequently translocated to North-Africa (Fig. 3, see also Dobson 1998). This

would neatly explain the seeming contradiction between nuclear and mitochondrial genetic data. The fact that the Mesola red deer do not show a mitochondrial haplotype unequivocally linked with those of the Tyrrhenian and the Barbary red deer is probably the result of drift in the small, long-time isolated and bottlenecked Mesola population. Effective population size is four times smaller for the mitochondrial than for the nuclear gene pool, which is why drift has a fourfold smaller effect on (nuclear) microsatellite allele frequencies than on control region sequences.

Apart from presenting a very probable solution to the debate about the origin of the Tyrrhenian red deer and its relative relationships with mainland Italian and African Barbary red deer, the study of Hajji et al. (2008) also highlights the need for taking into consideration all available sources – palaeontological, archaeozoological and genetic, and it is also a corroboration of previous studies, which warned against the exclusive use of mitochondrial DNA in phylogeographic analyses (e.g., Taberlet et al. 1998).

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# Phylogeographic Analyses of a Boreal-Temperate Ectomycorrhizal Basidiomycete, *Amanita muscaria*, Suggest Forest Refugia in Alaska During the Last Glacial Maximum

József Geml, Rodham E. Tulloss, Gary A. Laursen, Nina A. Sazanova, and D. Lee Taylor

**Abstract** Beringia, including Alaska and North-Eastern Siberia, has long been a focal point for biogeographical research in a wide range of plant and animal taxa. Whether boreal forest refugia existed in Beringia during the Last Glacial Maximum (LGM) is a major but unanswered question in Quaternary science. We analysed DNA sequence data from *Amanita muscaria* using phylogenetic and coalescent methods to test whether this boreal-temperate ectomycorrhizal fungus was present in Alaska during the LGM. Our data indicates that there are at least two endemic groups showing no detectable migration from or to populations that survived the LGM in Southern refugia. One of these occurs in the boreal forests of Interior Alaska, while the other group is endemic to the maritime rainforests from Southeast Alaska to the Olympic peninsula. These results suggest genetic isolation preceding the LGM and support the hypothesis of glacial forest refugia in Interior Alaska and along the coast of Southeast Alaska and British Columbia.

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## 1 Introduction

Climatic and geological changes in the Tertiary and Quaternary dramatically influenced the distribution of flora and fauna in North America. In the Tertiary, North America became separated from Eurasia, resulting in vicariant populations of many species of the ancient flora and fauna (Budantsev 1992; Graham 1999). During glacial maxima, plants, fungi and animals were forced to unglaciated refugia, from where they expanded to newly exposed areas in warmer interglacial periods. Reconstruction of these historical events is of paramount importance because they had major influences on past speciation events and are reflected in present day population structures.

Beringia, including Alaska and North-Eastern Siberia, has long been a focal point for biogeographical research in a wide range of plant and animal taxa. This high level of interest arises for two principal reasons. First, due to its diverse landscape and climate and the fact that much of the region remained ice-free during glacial maxima, Beringia served as a refugium for Arctic and subarctic flora and fauna (Adams and Faure 1997; Brubaker et al. 2005; Edwards et al. 2000; Hultén 1968). Second, during much of the Tertiary and the Quaternary periods, Beringia was the major land connection between Asia and North America and provided migration routes to a wide variety of organisms (for example, see Elias et al. 2000; Qian 1999; Swanson 2003). High genetic diversity and evidence for glacial refugia in Beringia have been reported in several, predominantly arctic-alpine animal and plant taxa, such as the Tundra Vole (*Microtus oeconomus*) (Brunhoff et al. 2003), Bighorn Sheep (*Ovis canadensis*) and Dall Sheep (*O. dalli*) (Loehr et al. 2005), Columbian Ground Squirrel (*Spermophilus columbianus*) (MacNeil and Strobeck 1987), Old World Swallowtail (*Papilio machaon*) (Sperling and Harrison 1994), *Amara alpina*, an arctic-alpine ground beetle (Reiss et al. 1999), *Paranoplocephala arctica*, a parasitic cestode of collared lemmings (Wickström et al. 2003), Entire-leaved Avens (*Dryas integrifolia*) (Tremblay and Schoen 1999), Purple Saxifrage (*Saxifraga oppositifolia*) (Abbott and Comes 2003), Arctic Bell-heather (*Cassiope tetragona*) (Eidesen et al. 2007) and the Bog Blueberry (*Vaccinium uliginosum*) (Alsos et al. 2005).

Whether fragments of boreal forest existed in Beringia during the Last Glacial Maximum (LGM) is a major, but, as yet, unanswered question in Quaternary science. Although earlier pollen-based biome reconstructions suggest that all of Beringia was covered by arctic tundra with no conifers (Edwards et al. 2000; Kaufman et al. 2004; Swanson 2003), phylogeographic analyses of DNA sequences and the most recent pollen data support the theory that *Picea glauca* and *P. mariana* existed in small refugia in Alaska (Anderson et al. 2006; Brubaker et al. 2005).

Ectomycorrhizal (ECM) fungi are obligate root-associated mutualistic symbionts of trees in boreal, temperate and tropical forests (Smith and Read 1997; Bas 1969; Härkönen et al. 1994; Simmons et al. 2002) and their past and present populations likely have been shaped by the distribution of their host trees. The likely importance of host trees in the distribution of ECM fungi has been repeatedly noted.

However, despite their ecological importance, very little is known about ECM phylogeography. Furthermore, learning about the phylogeographic history of ECM taxa not only provides information related to the organism in question, but may also help to resolve whether or not their hosts survived the LGM in Beringian refugia. To test the hypothesis of persistent LGM forest refugia in Alaska, we analysed molecular data from *Amanita muscaria*, an ECM fungus native to temperate and boreal forest regions of the Northern Hemisphere.

## 2 Materials and Methods

Multi-locus DNA sequence data for *A. muscaria* specimens were generated in previous studies (Geml et al. 2006, 2008, and Oda et al. 2004). Geml et al. (2006) reported that there are at least three phylogenetic species clades within the *A. muscaria* species complex that occur in Alaska (referred to as Clades I, II, and III). To our knowledge, Clade I is restricted to North America, while Clades II and III occur in Eurasia and in Alaska. For our tests in this study, 114 ITS rDNA sequences were chosen, which represent the three phylogenetic species. Because the phylogenetic species mentioned are non-interbreeding entities, population-level analyses were conducted separately for each species clade. Multiple sequence alignments were made using Clustal W (Thompson et al. 1997) and subsequently were corrected manually. Identical sequences were collapsed into haplotypes using SNAP Map (Aylor and Carbone 2003) after recoding insertion or deletions (indels) and excluding infinite-sites violations. The analyses presented here assume an infinite sites model, under which a polymorphic site is caused by exactly one mutation and there can be no more than two bases segregating. Base substitutions were categorised as phylogenetically uninformative or informative, and as transitions or transversions. Site compatibility matrices were generated from each haplotype dataset using SNAP Clade and SNAP Matrix (Markwordt et al. 2003) to examine compatibility/incompatibility among all variable sites, with four resultant incompatible sites removed from the data set. This was important as subsequent coalescent analyses assume that all variable sites are fully compatible. Two migration models were used. First, MDIV (Nielsen and Wakeley 2001) was used to determine whether there was any evidence of migration between Alaskan and non-Alaskan populations in each species clade, i.e. to test whether Alaskan populations could have survived the LGM in local forest refugia. For this purpose, specimens were assigned either to the 'Alaskan' or the 'non-Alaskan' group based on their localities. In Clades I and II, the 'non-Alaskan' groups consisted of specimens collected in the contiguous states of the US or in Eurasia, respectively. These represented populations that survived the LGM in Southern refugia. MDIV implements both likelihood and Bayesian methods using Markov chain Monte Carlo (MCMC) coalescent simulations to estimate the migration rate ( $M$ ), population mean mutation rate ( $\Theta$ ), divergence time ( $T$ ) and the time since the most recent common ancestor (TMRCA). This approach assumes that all populations descended from one panmictic population

that may or may not have been followed by migration. For each dataset, the data was simulated assuming an infinite sites model with uniform prior. We used 2,000,000 steps in the chain for estimating the posterior probability distribution and an initial 500,000 steps to ensure that enough genealogies were simulated before approximating the posterior distribution. Second, if MDIV showed evidence of migration, MIGRATE was used to estimate migration rates assuming equilibrium migration rates (symmetrical or asymmetrical) in the history of the populations (Beerli and Felsenstein 2001). We applied the following specifications for the MIGRATE maximum-likelihood analyses:  $M$  and  $\Theta$  generated from the FST calculation, migration model with variable  $\Theta$  and constant mutation rate. Subsequently, we reconstructed the genealogy with the highest root probability, the ages of mutations and the TMRCA of the sample using coalescent simulations in Genetree v. 9.0 (Griffiths and Tavaré 1994). Ages were measured in coalescent units of  $2N$ , where  $N$  is the population size.

### 3 Results

Two of the three species clades (Clades I and II) possessed sufficient variation in the ITS to test whether the Alaskan populations were genetically differentiated from non-Alaskan populations. The third, Clade III, was only represented by ten individuals forming three haplotypes with minimal variation. In this species, all Alaskan specimens carried the most frequent haplotype that was shared with Asian specimens as well. The datasets consisted of 653 and 640 characters for Clade I and Clade II, respectively, including gaps.

In Clade I, the ‘North American’ clade, 15 haplotypes were found, of which only one, haplotype D, was found in Alaska (Tables 1 and 2). MDIV showed no evidence of gene flow ( $M \sim 0$ ) and estimated a divergence time of  $T \sim 0.9$  between the Alaskan and non-Alaskan populations (Fig. 1a). Therefore, in simulations using Genetree, we assumed subdivided population structure and a very low level of migration (close to 0) for the starting backward migration matrix. The coalescent-based genealogy was informative with respect to the resolution of the splitting event between the Alaskan and non-Alaskan populations and inference of the mutational history with respect to variation between and within populations (Fig. 2a).

In Clade II, the ‘Eurasian’ clade, 14 haplotypes were found, six of which occur in Alaska (Tables 1-2). Of these six, five were found only in Alaska (haplotypes C, D, G, H, I), while one (haplotype A) represented the most abundant haplotype in the sample, spanning Eurasia and Alaska. It must be noted, that haplotype D (or subclade II/A in Geml et al. 2006), a lineage endemic to the maritime rainforests of the Pacific Northwest, was considered ‘Alaskan’ as opposed to ‘Eurasian’, despite not being strictly restricted by the political boundaries and occurring as far South as Northwestern Washington state. Apart from this, no other Clade II haplotypes have been found in North America, outside Alaska. Despite the very similar values for  $\Theta$  (Fig. 1), the population structure in Clade II proved to be very different from

**Table 1** Identity of *A. muscaria* haplotypes in our sample. Haplotype letters correspond to those in Table 2 and Fig. 2

Haplotypes	Specimen	Geographic origin
Clade I		
A	45785	Hampshire, Massachusetts, USA
B	45820	Bronx, New York, USA
C	CMP3143	Cochise Co., Arizona, USA
C	RET145-2	Tlaxcala, Mexico
C	CMP0648	Cochise Co., Arizona, USA
C	RET144-10	Tlaxcala, Mexico
D	GAL15330	Fairbanks, Alaska, USA
D	GAL16775	Fairbanks, Alaska, USA
D	GAL15776	Bonanza Creek LTER site, Alaska, USA
D	GAL16654	Fairbanks, Alaska, USA
D	GAL15336	Fairbanks, Alaska, USA
D	GAL17984	Fairbanks, Alaska, USA
D	GAL17982	Fairbanks, Alaska, USA
D	GAL15335	Fairbanks, Alaska, USA
E	RET145-1	Tlaxcala, Mexico
F	CMP1345	Cochise Co., Arizona, USA
G	49100	Cascade, Idaho, USA
H	45840	Lawrence, Massachusetts, USA
I	45843	Hampshire, Massachusetts, USA
J	45060	Amador, California, USA
J	LG382	Santa Cruz Island, California, USA
K	44761	Alpine, California, USA
L	45863	Mendocino, California, USA
L	RET320-1	Fremont Co., Idaho, USA
L	LG882	Santa Cruz Island, California, USA
M	RET271-2	Sussex Co., New Jersey, USA
N	45883	Piscataquis, Massachusetts, USA
N	RET271-3	Somerset Co., New Jersey, USA
O	RET383-3	Newfoundland, Canada
O	RET124-2	Suffolk Co., Massachusetts, USA
O	RET338-9	Clallam Co., Washington, USA
O	RET289-3	Cape May Co., New Jersey, USA
O	RET158-7	Burlington Co., New Jersey, USA
O	RET303-4	Monmouth Co., New Jersey, USA
Clade II		
A	30962	Kitakoma-gun, Yamanashi, Japan
A	30963	Kitakoma-gun, Yamanashi, Japan
A	30976	Kiso-gun, Nagano, Japan
A	30977	Ohno-gun, Gifu, Japan
A	30985	Ohno-gun, Gifu, Japan
A	30964	Gdynia, Poland
A	31445	Surrey, England, UK
A	80048	Surrey, England, UK

(continued)

**Table 1** (continued)

Haplotypes	Specimen	Geographic origin
A	N10	Magadan district, Magadan region, Russia
A	GAL14284	Denali National Park, Alaska, USA
A	GAL16735	Fairbanks, Alaska, USA
A	GAL5895	Serpentine Hot Springs, N. of Nome, Alaska, USA
A	GAL15453	North Pole, Alaska, USA
A	GAL15461	North Pole, Alaska, USA
A	GAL5505	Denali National Park, Alaska, USA
A	RET036-3	Zürich canton, Switzerland
A	RET309-3	Sogn og Fjordane, Norway
A	RET143-5	Kamchatka, Russia
A	RET141-2	Highlands & Islands Reg., Scotland, UK
A	N3	Ola district, Magadan region, Russia
A	N12	Ola district, Magadan region, Russia
A	N5	Magadan district, Magadan region, Russia
A	N13	Omsukchan district, Magadan region, Russia
A	N6	Magadan district, Magadan region, Russia
A	MP23	Bird Creek Campground, Anchorage, Alaska, USA
A	N15	Tenka district, Magadan region, Russia
A	MP24	Bird Creek Campground, Anchorage, Alaska, USA
A	N16	Anadyr district, Chukot Autonomous Region, Russia
A	N1	Ola district, Magadan region, Russia
A	N11	Ola district, Magadan region, Russia
A	GAL8950	Denali National Park, Alaska, USA
A	GAL5900	Serpentine Hot Springs, N. of Nome, Alaska, USA
A	GAL5946	Quartz Creek, E. of Nome, Alaska, USA
A	GAL16735-2	Fairbanks, Alaska, USA
A	GAL18071	Fairbanks, Alaska, USA
A	GAL16735-3	Fairbanks, Alaska, USA
A	GAL18076	Fairbanks, Alaska, USA
A	GAL15454	North Pole, Alaska, USA
A	GAL18134	Bonanza Creek LTER site, Alaska, USA
A	GAL18136	Bonanza Creek LTER site, Alaska, USA
A	GAL17899	Fairbanks, Alaska, USA
A	GAL18012-6	Fairbanks, Alaska, USA
A	GAL18012-2	Fairbanks, Alaska, USA
A	GAL18012-4	Fairbanks, Alaska, USA
A	GAL18810-2	Homer, Alaska, USA
A	GAL18810-1	Homer, Alaska, USA
A	GAL17691	Fairbanks, Alaska, USA
B	RET107-6	Highlands & Islands Reg., Scotland, UK
C	GAL2005	Bonanza Creek LTER site, Alaska, USA
D	RET264-7	Skamania Co., Washington, USA
D	GAL4247	Glacier Hwy, mile 27, Juneau, Alaska, USA
D	GAL4302	Glacier Hwy, mile 27, Juneau, Alaska, USA
D	RET136-2	Whatcom Co., Washington, USA

*(continued)*

**Table 1** (continued)

Haplotypes	Specimen	Geographic origin
D	GAL3643	Glacier Hwy, mile 30, Juneau, Alaska, USA
E	RET149-1	Southern Highlands Prov., Tanzania
E	RET149-2	Southern Highlands Prov., Tanzania
F	N8	Magadan district, Magadan region, Russia
G	GAL18122	Bonanza Creek LTER site, Alaska, USA
H	GAL2814	Dalton Hwy, mile 122, Alaska, USA
I	GAL4810	Denali National Park, Alaska, USA
J	31452	Hampshire, England, UK
J	30965	Gdansk, Poland
J	RET152-6	Baden-Württemberg, Germany
J	RET152-8	Bavaria, Germany
K	30961	Aomori-shi, Aomori, Japan
L	30987	Queenstown, New Zealand
M	N4	Ola district, Magadan region, Russia
N	N14	Tenka district, Magadan region, Russia

that of Clade I discussed above. MDIV showed evidence for intermediate levels of gene flow ( $M \sim 2.5$ ) between Alaskan and Eurasian populations and estimated no population divergence ( $T \sim 0$ ) (Fig. 1b). Our analyses using MIGRATE suggested that migration between Alaska and Eurasia has been asymmetrically bidirectional with Alaska receiving more migrants ( $M_{12} = 9,482.98$  and  $M_{21} = 33.53$ ;  $M_{12}$  referring to the migration from Eurasia to Alaska, and  $M_{21}$  *vice versa*). Therefore, in simulations using Genetree, we assumed panmictic population structure. As expected, the coalescent-based genealogy did not show any historical population division (Fig. 2b). However, based on the fact that the specimens from the maritime rainforest ecoregion of the Pacific Northwest of North America formed a well-supported sister group (II/A) to the rest of Clade II in our previous multi-locus phylogenetic analyses (Geml et al. 2006, 2008), we tested in a separate analysis whether or not there was any gene flow between this group inhabiting a special habitat with a restricted distribution and the rest of Clade II, this latter including boreal Alaskan and Eurasian specimens. In this case, MDIV showed no migration ( $M \sim 0$ ) and estimated a divergence time of  $T \sim 1.5$  between the two populations, i.e. subclade II/A vs. Clade II (Fig. 1c). Nonetheless, the coalescent-based genealogy estimated under an assumption of population subdivision did not differ significantly from the one generated assuming panmixia (Fig. 2c).

## 4 Discussion

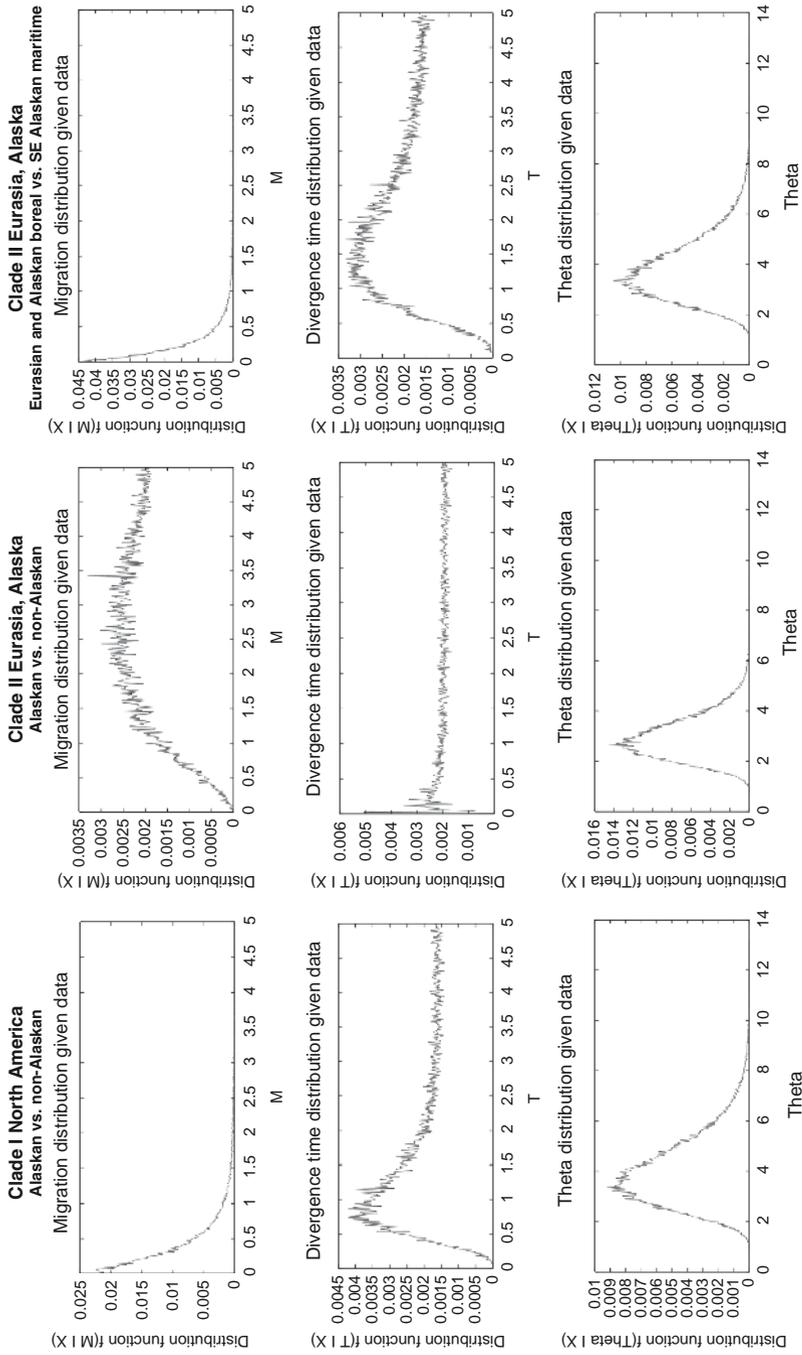
The main goal of this project was to estimate migration between Alaskan and non-Alaskan populations within distinct phylogenetic species in the *A. muscaria* species complex in order to test whether or not these fungi could have survived the

**Table 2** Polymorphic sites in haplotypes collapsed after recoding indels and excluding infinite-sites violations from the original dataset for coalescent analyses. Position refers to that in the original alignment, site number is the designation of the given mutation as shown on Fig. 1, site type refers to transition (t), transversion (v), or deletion (-) change with regard to the consensus sequence. Character type indicates either parsimony-informative (i) or non-informative (-) sites. Capital letters refer to haplotype designations on Fig. 2

Phylogenetic species	Clade I	Clade II
Position	1111122223344455 16660136600132712668 00134960218778400483	1111111233456 2446672334558705061 366231200182485654280
Site number	11111111112 12345678901234567890	111111111122 123456789012345678901
Site Type	tvt-tttttttttt	tttttttvtvvvtvtt
Character Type	-i-i-ii-----i---	-----i--i-----
Haplotypes:		
A	.....C...	.....
B	T.2.....	G.....CT.....
C	.T..T.....	.G.....
D	.T.....C	.GC.CA.....T.
E	.T..T.C.....	...T..T...CC.....
F	.T..T..T...G.A..	.....C.....
G	..A..2.....T...	.....T.....
H	...2.....	.....A.....
I	...A.....	.....C.A.....
J	.....2.....T...	.....T.....
K	.....2.G..G.C.....	.....A.....
L	.....2.....	.....A.....
M	.....T.G.....	.....A.....
N	.....T.....	.....A.....
O	.....	.....

LGM in Alaska. Because non-Alaskan populations most likely survived the LGM in refugia South of the major ice shields, the lack of migration between these and the Alaskan ones would suggest local survival of the latter, implying forest refugia in Alaska.

Overall, we found support for at least two endemic regional populations (haplotype D in Clade I, and haplotype D in Clade II) in different parts of Alaska, both of which exhibited genetic isolation and differentiation from other conspecific populations. Unfortunately, we could not estimate the times of divergence of the clades with certainty due to the lack of fossils and the great variance in nucleotide substitution rates in fungi. Nonetheless, it seems very likely that the major lineages (Clades I-III) separated well before the Pleistocene glacial cycles (Geml et al. 2006). On the other hand, the endemic regional populations likely represent groups that became isolated from the rest of their species in the Pleistocene and survived one or more glacial maxima in local refugia. Our findings support the existence of at least two independent such glacial forest refugia: (1) boreal forest in Interior Alaska; and

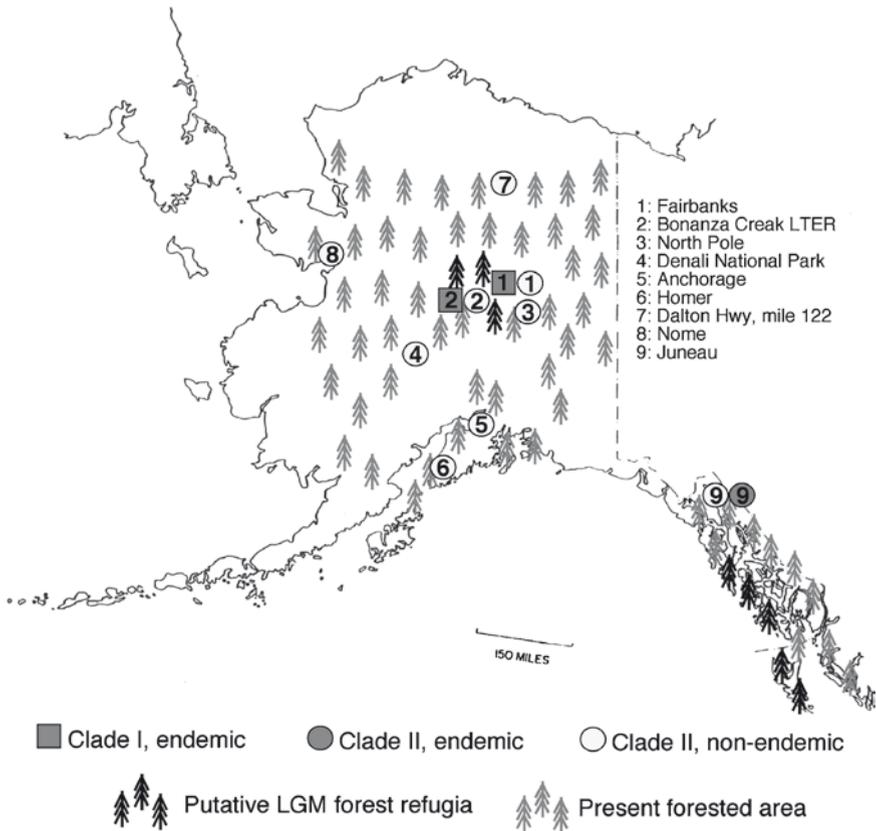


**Fig. 1** Posterior probability distributions of migration ( $M$ ), divergence time ( $T$ ) and population mean mutation rate ( $\Theta$ ) between population pairs using MDIV. Estimates (axis X) with the highest distribution function ( $\Theta$ ) values have the greatest probabilities. A and C show examples of zero migration and non-zero divergence time, while graphs in B indicate recent gene flow and no divergence between population pairs



(2) maritime rainforest in Southeast Alaska and the Pacific Northwest (Fig. 3). Apart from this, many Alaskan haplotypes in Clade II were shared with specimens from Eurasia. Our results suggesting high migration, most likely both pre- and post-glacial, across the Bering Strait and the similar observed numbers of haplotypes endemic to either Eurasia or Alaska leave open the question whether boreal members of Clade II survived the LGM in Alaska or colonised Alaska from Asia in multiple times in postglacial events.

The possible existence of forest refugia in Interior and Southeast Alaska is also supported by several other independent lines of evidence. For example, although previous palynological biome reconstructions suggest that all of



**Fig. 3** Outline map of Alaska showing the collecting locations for the 43 Alaskan specimens included in this study. *Dark grey squares* and *circles* indicate sampling localities for endemic haplotypes, while *light grey circles* refer to haplotypes shared between Alaska and Eurasia. Black stylised spruce drawings mark putative forest refugia during the Last Glacial Maximum (LGM) suggested by this and other studies mentioned in the discussion. *Grey tree figures* indicate present forested area, without making a distinction between boreal and maritime forests of Interior and Southeast Alaska, respectively

Beringia was covered by arctic tundra (Edwards et al. 2000; Kaufman et al. 2004; Swanson 2003), recent pollen data by Brubaker et al. (2005) supports the theory that *Picea* forests were present in Eastern Beringia, at least in small fragments restricted to habitats with favourable microclimates. The Northernmost distribution of *Picea*, unlike that of *Betula* and *Populus*, is restricted, by definition, to the boreal region. Therefore, Brubaker's findings indicate the existence of the boreal forest biome in Alaska during the LGM. Similarly, Anderson et al. (2006), surveying forest stands across Northwestern North America, found several chloroplast DNA haplotypes of *Picea glauca* that were unique to Alaska, suggesting local survival. Maroja et al. (2007) analysed mitochondrial DNA sequence data from the spruce beetle (*Dendroctonus rufipennis*) and suggested that the postglacial range expansion of these beetles occurred from three refugia, one of which was in Interior Alaska.

Forest refugia in Southeast Alaska are also supported by genetic studies in several different taxa. For example, this is a region with high mammalian endemism due to its fragmented landscape and complex glacial history. Even animals, as mobile as wolves, have been shown to exhibit a strong signal of independent histories for the coastal and continental populations (Weckworth et al. 2005). Also, phylogenetic studies of Fleming and Cook (2002) on ermine (*Mustela erminea*) showed that there was at least one lineage strictly endemic to Southeast Alaska that likely survived locally during the Wisconsin glaciation. Locations for such glacial refugia in Southeast Alaska have been proposed by Carrarra et al. (2003) based on geological evidence.

In this paper, we demonstrated the use of ECM fungal taxa to indirectly infer glacial vegetation history. Therefore, our results are not only relevant to the mycological community, but to a broader biological community in general, particularly to those with a biogeographic interest.

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# Review

## Refugial Areas and Postglacial Colonizations in the Western Palearctic

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### 1 Introduction

Past climatic oscillations, such as the glacial-interglacial cycles of the Pleistocene (Williams et al. 1998) led to severe worldwide altitudinal and latitudinal range shifts of taxa and ecosystems (Hewitt 2004). For a reconstruction of the glacial and postglacial history of species, data obtained from methods ranging from evolutionary genetics to the analysis of distribution patterns and studies of pollen and subfossil remains of invertebrates and vertebrates are essential (de Lattin 1967; Coope 1970, 1978, 1994; Varga 1977; Huntley and Birks 1983; Taberlet et al. 1998; Willis et al. 1995; Hewitt 1999, 2000; Willis and van Andel 2004; Schmitt 2007; Varga and Schmitt 2008). The synthesis of all these analyses reveals the existence of different paradigm patterns in the Western Palearctic. In the following, we give a brief overview of these patterns.

### 2 Mediterranean Glacial Refugia

During the glacial periods, most thermophilic species became extinct over major parts of their former distribution range and only survived in “refugia” at lower latitudes which presented suitable climatic conditions. In Europe, these areas, located South of the transversal European high mountain systems, are the three well-studied refugial regions of the Mediterranean peninsulas (Iberia, Italy, and Balkans), all

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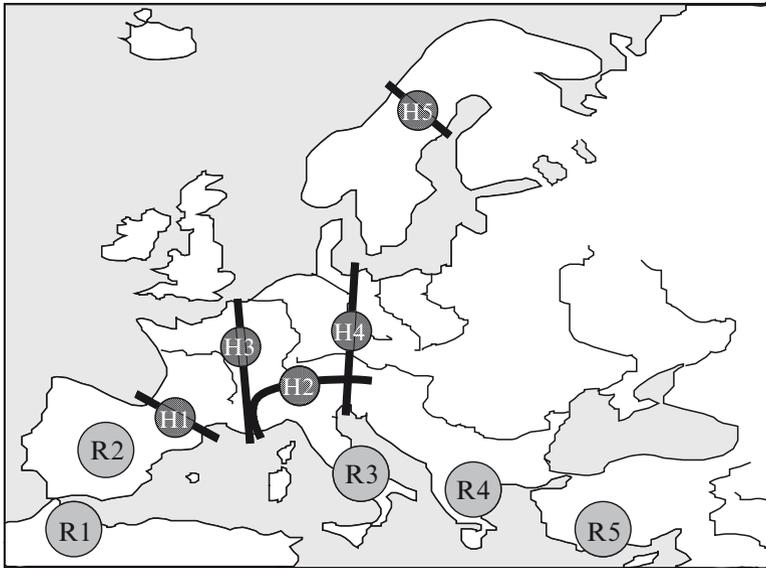
more or less isolated from each other (Reinig 1937; de Lattin 1949; Huntley and Birks 1983; Bennett et al. 1991; Comes and Kadereit 1998; Hewitt 1996, 1999; Taberlet et al. 1998). The Maghreb and Asia Minor represent two further important areas in which thermophilic organisms survived the glacial periods (Busack 1986; Seddon et al. 2001, 2002; Harris et al. 2002; Seddon et al. 2002; Cosson et al. 2005; Fritz et al. 2006; Horn et al. 2006; Marmi et al. 2006; Weingartner et al. 2006; Habel et al. 2008). During these glacial periods, species differentiated into distinct genetic lineages, and repeated allopatric disjunctions reinforced these genetic differentiations, sometimes resulting in speciation events (Santucci et al. 1998; Habel et al. 2005). Subcenters have been postulated for these Mediterranean refugia (e.g., Reinig 1950); these have been tested by recent molecular analyses for the Balkans (Poulakakis et al. 2003, 2005; Parmakelis et al. 2006a, b Schmitt et al. 2006), Iberia (Harris et al. 2004; Martínez-Solano 2004; Carranza et al. 2006b), Southern Italy (Steinfartz et al. 2000; Seddon et al. 2001; Podnar et al. 2005; Canestrelli et al. 2006), and the Maghreb (Cosson et al. 2005; Carranza et al. 2006a,b; Fritz et al. 2006).

In the wake of climate warming during the postglacial period, the species expanded their distribution ranges from these refugia into higher altitudes and latitudes. These movements generally followed four paradigm patterns (Hewitt 2000; Habel et al. 2005): (1) postglacial expansion from all three Southern European differentiation centers, (2) expansion of the Western and Eastern lineages, with the Adriatic–Mediterranean lineage being trapped in Italy by the Alps, (3) expansion of the Adriatic– and Pontic–Mediterranean lineages, but trapping of the Atlantic–Mediterranean lineage by the Pyrenees, and (4) major expansion to Central Europe only from the Balkans, and trapping of the Atlantic– and Adriatic–Mediterranean lineages by the Pyrenees and Alps, respectively. These patterns are frequently repeated in many animal and plant species.

These recolonizations resulted in secondary contact and hybrid zones which can be traced by sensitive molecular markers (Hewitt 1988, 1993). Five regions of Europe are known for their high number of hybrid zones: (1) the Alps, (2) the Pyrenees, (3) Western Central Europe, (4) Eastern Central Europe, and (5) central Scandinavia (Taberlet et al. 1998; Hewitt 1999; Schmitt 2007). Further processes which occurred during colonization processes, such as the loss of genetic diversity due to stepping stone or leptokurtic range expansion, can be identified that help in the interpretation of the mode of past range change dynamics and the core areas of historical distribution areas (Hewitt 2001) (Fig. 1).

### **3 Extra-Mediterranean Refugia of Cold-Adapted and Temperate Species**

During glacial periods, the regions between the Alps, Pyrenees, and Balkan mountains in the South and the Northern European ice shield in the North were largely covered by tundra and cold steppe on permafrost. Only cold-adapted species were



**Fig. 1** The five major Mediterranean refugia and differentiation centers of the South European peninsulas (R1: Atlantic-Mediterranean in the Maghreb, R2: Atlantic-Mediterranean in Iberia, R3: Adriatic-Mediterranean, R4: Pontic-Mediterranean at the Balkans, R5: Pontic-Mediterranean in Asia Minor) and secondary contact- and hybridization zones during the postglacial range expansion (H1: Pyrenees, H2: Alps, H3 Western Central Europe, H4 Eastern Central Europe, H5: central Scandinavia). After Schmitt (2007), modified

able to survive under these extreme conditions. Many of these species are now restricted to the high mountain systems and/or the high latitudes of Eurasia, though they sometimes even reached North America (Holdhaus and Lindroth 1939; Holdhaus 1954; Varga and Schmitt 2008). Some, however, also have relict populations in cold habitats of lower mountain ranges. Stony debris formations with their exceptionally cold microclimatic conditions (at some sites with permafrost ground) host such species (Molenda 1996). Some of these species live exclusively North of the Alps (and not in the Alps!), thus making them Central European endemics (e.g., the ground beetle *Pterostichus neglectus*). However, many typical mountain species also showed discontinuous distribution patterns around the predominantly glaciated high mountain systems during the ice ages.

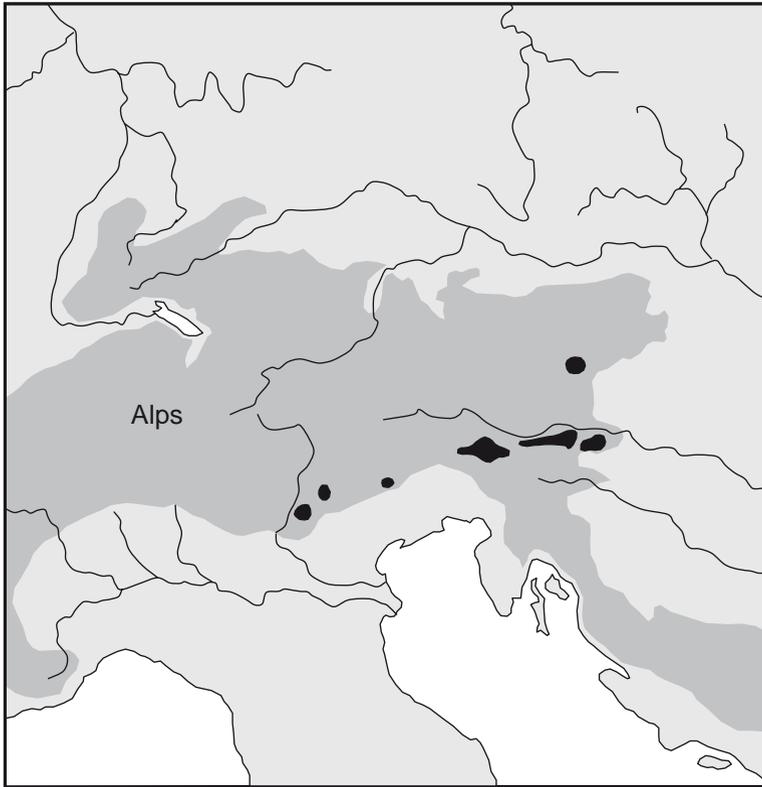
Furthermore, growing evidence supports the survival of numerous species which require woodlands or temperate climatic conditions North of the Mediterranean refugia scattered over large areas of Central Europe (Englbrecht et al. 2000; Jaarola and Searle 2002; Babik et al. 2004; Fink et al. 2004; Pinceel et al. 2005). This mainly coincides with the detection of woodland or at least tree species refugia in Central Europe during glacial periods (e.g., Willis and van Andel 2004). The Black Forest (and/or an area in its vicinity), for example, is discussed by Fink et al. (2004) and Drees et al. (2009) as a possible glacial refugium for woodland dwelling species.

Phylogeographic studies on some vertebrates supported the existence of previously unknown, or cryptic, refugia (e.g., in the Carpathians). The genetic differentiation of seaweeds, coupled with the concordance of similar patterns across these species, gives reason to conclude that some marine species persisted through the last ice age in flooded depressions on the floor of the present-day English Channel (see Provan and Bennett 2008 for a review of these cryptic refugia). Using an integrative approach with both fossil and genetic data, Petit et al. (2008) concluded that some temperate and boreal trees apparently survived the last glacial maximum under periglacial conditions, probably close to ice sheets and even in unglaciated areas North of ice sheets. All these recent results are beginning to revolutionize our understanding of glacial refugia for cold-adapted and temperate species. Analyzing the species distribution models with moderate to good predictive ability, Svenning et al. (2008) concluded that the view of Central European landscape North of the Alps as largely treeless needs to be revised.

#### 4 “Massifs de Refuge” at the Southern Margin of the Alps

The former heavily glaciated inner regions of the Alps are highly differentiated from the Southern and Southeastern margins of these mountains, which were not strongly glaciated during the glacial periods. The latter regions are today characterized by an extraordinarily high biodiversity (cf. Ozenda 1982; Varga and Schmitt 2008). These glacial refugia at the Southern margin of the Alps have been described as “Massifs de Refuge” by botanists and zoologists lasting for over a century (e.g., Chodat and Pampanini 1902; Holdhaus 1904). Similar phenomena are also known for the Jura, the Northern Kalkalpen, and also large parts of the Pyrenees and Carpathians. Blind cave species were able to survive in these refugia (Assmann et al. 2009), and many other species, both plants and animals, with highly restricted distribution areas were scattered along the Southern and Southeastern margin of the Alps. Because some glaciers extended into the lowlands of Northern Italy, the Massifs de Refuge were interrupted and therefore lined up like “pearls on a necklace”. Many of the species restricted to these areas are true relict species which had wider ranges in the past – as evidenced by their recent disjunct distribution pattern (e.g., *Pterostichus zieglerti*, Fig. 2).

On the other hand, some species which survived in these Massifs de Refuge also (re-)colonized the previously glaciated areas as e.g. known from weevils and moths. These species have two types of populations, diploid (with sexual reproduction) and polyploid (with parthenogenetic reproduction), with most species belonging to the genera *Otiorrhynchus* and *Solenobia* (Jahn 1941; Seiler 1943, reviewed in Suomalainen et al. 1976). The bisexual populations, from which the parthenogenetic populations evolved, represent the plesiomorphic group. In some weevil species, the diploid populations are mostly restricted to the regions of the Massifs de Refuge, and the polyploid populations exist in the parts of the Alps which were covered by ice sheets during the glacial periods, and/or in Central and Northern Europe. Thus, it is most likely that polyploidy evolved during postglacial range expansions.



**Fig. 2** Distribution pattern of the relict species *Pterostichus zieglerei* in the “Massifs de Refuge” at the Southern border of the Eastern Alps. After Holdhaus (1954) and Magistretti (1965), modified. Grey: Elevations above 500 m

## 5 Glacial Refugia in Northern Europe

Northern Europe with only few ice-free areas during the glacial periods appears to be an unlikely refugial area. However, some strongly differentiated species with several subspecies occur in Northern Europe, and at least some of these species were related to areas unglaciated during the ice ages. Consequently, Lindroth (1968, 1969, 1970) developed the hypothesis that some species survived the last glacial period in ice-free areas of Scandinavia. The majority of his contemporaries rejected this hypothesis. Evidence from recent genetic studies on rock ptarmigans (*Lagopus mutus*) and lemmings (*Lemmus lemmus*), however, supports the hypothesis of glacial refugia in Northern Europe (e.g., in Iceland and Northwestern Norway; Holder et al. 1999; Federov and Stenseth 2001). Thus, the existence of whole ecosystems with plant and animal communities seems probable, although more intensive investigations, especially in the field of strongly differentiated species are needed to confirm this.

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# Multiple Glacial Refuges of Unwinged Ground Beetles in Europe: Molecular Data Support Classical Phylogeographic Models

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**Abstract** Since the 1930s, several European zoologists have developed scenarios for glacial refuges and postglacial expansions, mainly based on studies of the morphological differentiation of populations and distribution patterns of species. For example, Holdhaus described the distribution of blind euedaphic and troglotic beetles restricted to an area South of a well-defined line crossing the Southern Europe from West to East. In these areas, where many endemic animal and plant species occur, other species that are currently more widely distributed in Europe were probably able to survive the glacial period(s). Molecular analyses of 77 populations of the silvicolous ground beetle *Carabus auronitens* support the existence of these postulated refuge areas. Genetic differentiation of *C. auronitens* provides good evidence for multiple refuges, which are, however, situated further North than previously assumed. Furthermore, genetic differentiation is more pronounced in the areas South of the “Holdhaus line” than in the areas North of it.

## Dedication

We dedicate this work to Prof. em. Dr. Friedrich Weber, our former academic supervisor and colleague, without whose tenacity in the study of *C. auronitens* this paper would not have been possible.

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## 1 Introduction

The Evolutionary Synthesis of Dobzhansky, Mayr, Rensch, Wright, Simpson, and others communicated the idea of population genetics to other fields of biology. These authors argued convincingly that mutation, recombination, selection, and gene flow operating within species (“microevolution” in Dobzhansky’s term) account for the origin of new species and for the long-term effects of evolution (“macroevolution” in Dobzhansky’s term) (Dobzhansky 1937). The European exponent of Evolutionary Synthesis, Bernhard Rensch (Futuyma 2005), was very interested in the processes of genetic drift as can be studied easily in small populations, such as on islands and in glacial refuges (Rensch 1929, 1939, 1951). A lively discussion on the processes of differentiation, leading to racial formation and speciation, was held in these years (Rensch 1929, 1939, 1960; Krumbiegel 1932, 1936; De Lattin 1959). While Krumbiegel (1936) described and measured morphological differences as a result of these processes, the genetic consequences of the climate-induced range shifts, including postglacial recolonization, were hypothesized for the first time by Reinig (1938, 1939). Recently, this idea was confirmed by compilations of studies using molecular data (Hewitt 1996, 1999; recent review by Schmitt 2007).

Biogeography profited from the development of both evolution-orientated population biology and the classical models of biogeography by pioneers, such as Holdhaus (Holdhaus and Lindroth 1939) and De Lattin (1957, 1959, 1967). Holdhaus showed that the distribution of blind euedaphic and troglobitic (cave-inhabiting) beetles is restricted to a well-defined area in Europe (Holdhaus 1954). In the South of the “Holdhaus line”, defined by the Northern distribution limit of these species, many endemic species occur. These areas, which coincide with the occurrence areas of those for endemic plant species of the Alps, have been known as “Massifs de Refuge” after Chodat and Pampanini (1902), and were discussed by Holdhaus (1906) as refuge areas also suitable for animals.

Surprisingly, Holdhaus’ ideas on glacial refuges and postglacial colonization processes have never been rejected although contemporary Anglophone scientific work and biogeography primers have mostly ignored them. One reason for this might be that these basal works have mainly been published in German.

Over the last decades, our knowledge of processes of glacial and postglacial periods has increased enormously, mainly due to the progress in evolutionary genetics and the use of a variety of genetic markers (e.g., Stauffer et al. 1999; Seddon et al. 2001; Michaux et al. 2003; Berggren et al. 2005; Joger et al. 2007) and the combination of these results with other methods (e.g., analysis of pollen and fossil records) to reconstruct the past events (Terhurne-Berson et al. 2004; Cheddadi et al. 2006; Magri et al. 2006; Magri 2008). Interestingly, not only historical but also recent hypotheses and postglacial recolonization scenarios are frequently based on studies of insect species, especially on those with low dispersal power (e.g., Hewitt 1996).

The aims of our study are to test whether

1. the Holdhaus line is congruent with the current knowledge on the distribution of endemics in Europe. We use ground beetles as a taxonomic group for this reevaluation as they are well studied in Europe at most relevant levels (especially faunistics and taxonomy, Assmann et al. 2008). This beetle family comprises a vast number of species in Europe (over 3,600), many of which are blind. Thus, this taxon also seems appropriate to test the validity of Holdhaus' biogeographic description of the Northern limit of blind beetles.
2. the Holdhaus line can be used for analytical procedures in present-day phylogeography of European species which aim to distinguish between relict and (re-)colonizing species (and populations), and to delimit glacial refuges. We use the stenotopic flightless woodland ground beetle species *Carabus auronitens* as a model species.

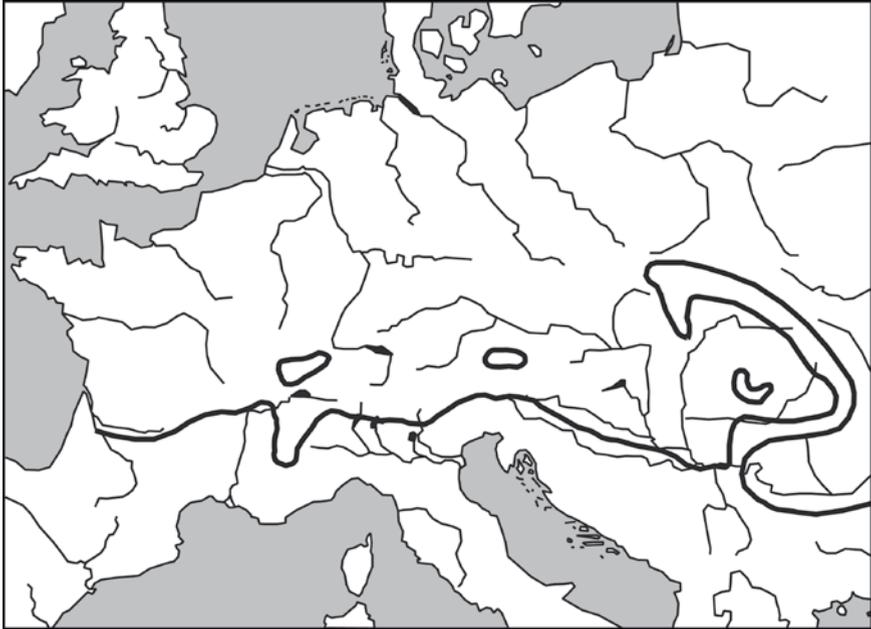
## 2 The “Holdhaus Line”: A Biogeographically Important Border in Europe

### 2.1 The Concept

Caves are very special habitats for animals due to their constant temperature and humidity conditions, their permanent darkness, and very low densities of prey. Consequently, troglobitic species often show striking morphological (long appendices, reduced eyes) and physiological (reduced metabolism, long life-cycles) adaptations to these habitats. Their dispersal ability is extremely low and limited. Most of the troglobitic species lack the ability to leave the massifs in which they occur and are, thus, among the species with the lowest power of dispersal (Culver and White 2005). In situations with such pronounced isolation between populations, numerous species were developed. For example, the world diversity hotspot of troglomorphic genera of Trechini (a species-rich tribe of ground beetles with many anophthalmic (eyeless) species) is located in the Western Palearctic realm (more than 50 genera) as compared with Asia (more than 30), the Nearctic (more than 15), and the Neotropics and Australia (about four and seven genera, respectively) (Casale et al. 1998; see also Lorenz 2005 for additional records on blind ground beetle genera in Southern Asia).

Holdhaus mapped the distribution ranges of two groups of beetles: the true cave-dwelling (troglobitic) beetles and the blind endogeic beetle species. He drew lines North of the distribution ranges of both groups, and stated that because of the very poor dispersal power of these species North of this border none of them could have survived glacial periods. We reevaluate the congruence between the Holdhaus line (as published in 1954) and our knowledge on Northern limits of cave and endogeic beetles.

Most of the cave-dwelling beetles inhabit not only caves but also systems of crevices and lacunae. In karstic regions, where most such species occur, they



**Fig. 1** Northern boundary of distribution ranges of anophthalmic cave beetle taxa in Europe: Holdhaus line. Modified after Holdhaus (1954)

frequently inhabit even whole massifs. Juberthie calls this special habitat “superficial underground compartment” (SUC) (Juberthie et al. 1980a,b), pointing out that there is no ecological difference between the so-called troglotic and the so-called anophthalmic and endogeic species. Indeed, extraordinarily large blind trechine beetles which have been regarded as true cave-dwelling species were also recorded from the SUC (Drovenik et al. 2008).

For this reason, it seems appropriate to combine both the lines described by Holdhaus (which are mainly congruent) in the “Holdhaus line”, named after its discoverer. The Holdhaus line runs from Bordeaux, via Lyon, the South of Alps, the Carpathians to the Black Sea (Fig. 1) (Holdhaus 1954) and is in good accordance with the Southern permafrost limit during the last glacial period (Lang 1994; Koenigswald 2002).

## 2.2 *The Holdhaus Line: Still up-to-Date?*

Since Holdhaus’ basic work, numerous additional species and a vast number of additional records of previously described endogeic and cave-dwelling species have come to be known (e.g., Löbl and Smetana 2003). We analyzed the faunistic and taxonomic literature (e.g., Bonadona 1971; Vigna-Taglianti 1982; Kryzhanovskij et al. 1995; Avon 1997; Löbl and Smetana 2003; Casale and Vigna-Taglianti 2005) and verified the geographic information of the records regarding potential deviations from Holdhaus’ concept.

In general, we found a remarkable congruence between the new findings and the “old” Holdhaus line. This is true for most faunistic records of anophthalmic or microphthalmic (with reduced eyes) ground beetles from a wide range of subfamilies and tribes (e.g., Trechini, Bembidiini, Anillina, Pterostichini, Platynini). We detected only three exceptions:

1. In the Northeastern Alps (Austria) and Northeast of the continuous line indicated by Holdhaus, there is an area with records of blind beetles. New records of the genus *Arctaphaenops* are outside the area marked by Holdhaus (cf. the records listed by Daffner 1993). The range of the three species which are regarded as valid species seems to be a little larger than the refuge area proposed by Holdhaus. This may be due to the postglacial expansions of the colonized area over distances of a few kilometers.
2. A similar situation exists in the Western Alps and its foothills (incl. the Jura) where the genera *Agostinia* and *Trichaphaenops* exhibit few populations (and species) (Bonadona 1971; Marggi 1992). We suggest uniting the isolated enclave North of the continuous Holdhaus line to an isolated range of an insignificant larger area (Fig. 1, Northwestern enclave).
3. Two records of blind carabid beetles are also known from sites far North of the Holdhaus line in Belgium (Desender 1986) and Germany (Malzacher 2000). Both records were taken in parks and gardens within cities. More than a century ago, trees (particularly, plane and chestnut) from Southern France were planted at both sites known to host blind endogeic Anillina beetles (*Anillus caecus*). Thus, it is very likely that the beetles were imported from the Mediterranean region along with earth and roots of young trees. Moreover, the survival of beetles proved the fact that they have the potential to survive far away from their usual distribution area and that their dispersal is limited.

These data support an extensive concordance between the distributions of blind species known today and those known in Holdhaus' time. Only very few corrections are needed to update the Holdhaus line.

A preliminary check of the distribution data of anophthalmic beetles from other families (e.g., Curculionidae, Cholevidae) supports the findings based on ground beetles, so that the Holdhaus line stands up to this initial perusal (cf. Casale et al. 1991; Giachino et al. 1998; Osella and Zuppa 1998).

### 3 The Holdhaus Line as a Tool for the Localization of Refuge Areas? The Case of *C. auronitens*

In this analysis, we investigate the hypothesis that the Holdhaus line helps to locate glacial refuges of species which are much more widely distributed today. As the Holdhaus line was derived from the distribution patterns of cave and endogeic beetles, this hypothesis may at least hold true for species of cold and humid habitats. We test this by compiling and reanalyzing the existing data from different population genetic analyses of *C. auronitens*.

### 3.1 *C. auronitens*

*C. auronitens* Fabricius, 1792 is a flightless ground beetle species of mostly cool and humid woodland habitats in both lowlands and montane to subalpine regions. Its distribution range covers wide areas of temperate Central Europe (Turin et al. 2003), with the Southernmost populations found in the Pyrenees, the Cevennes, Eastern parts of the Alps, and the Carpathians; it thus lives on either side of the Holdhaus line. In the Southwestern part of its range, the distribution is disjunct as populations exist in several areas in the Pyrenees (Forel and Leplat 1995) and the mountains further North (Montagne Noire and Cevennes, Puisségur 1964).

Both the biology and long-term population dynamics of the species (e.g., Weber and Heimbach 2001; compilation by Turin et al. 2003) and the genetic differentiation over its entire range (Assmann et al. 1994; Assmann and Weber 1997; Reimann et al. 2002) were investigated in detail.

### 3.2 Testing the Hypothesis

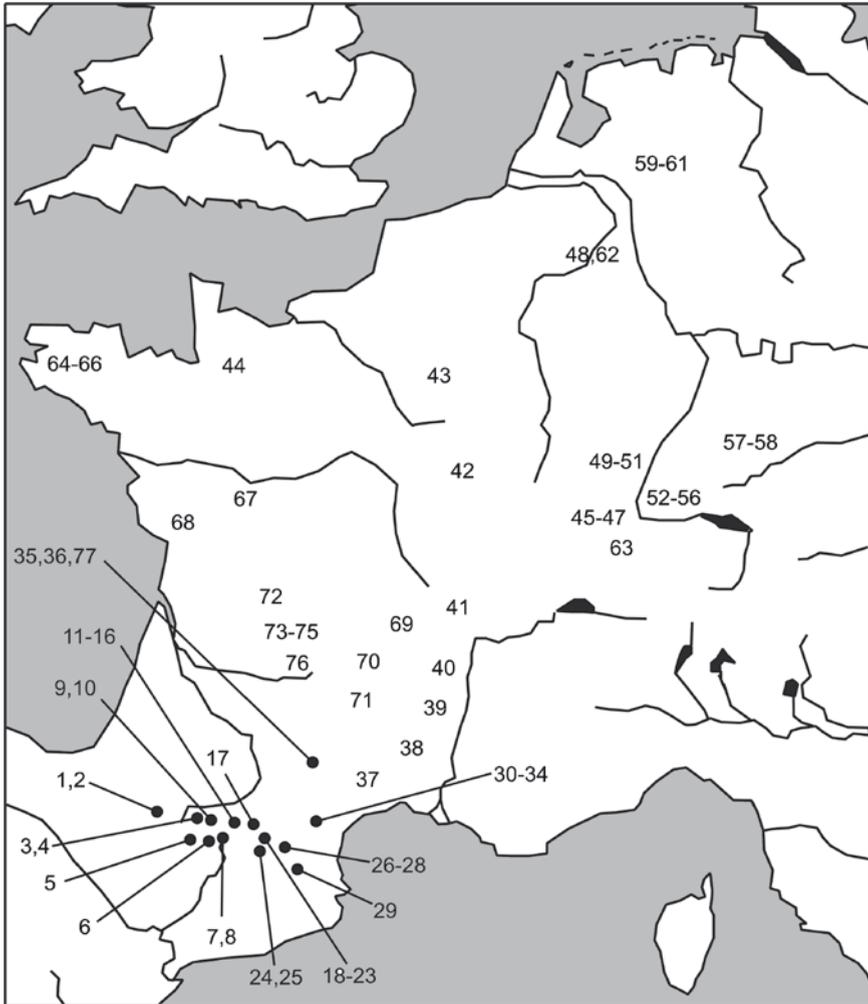
#### 3.2.1 Datasets

In order to test our hypothesis, we reanalyzed the population genetic data by Assmann et al. (1994), Assmann and Weber (1997), and Reimann et al. (2002). Allozyme polymorphisms at five loci (Table 1) were obtained by electrophoretic separation using acrylamide gels. Standard methods (as described in Assmann and Weber 1997) and consistent naming of the alleles (Reimann et al. 2002) allowed data compilation and reanalysis. Only samples with a minimum sample size of 15 individuals were used. We used two different datasets (Table 1). Dataset A contains

**Table 1** Population genetic datasets used for the reanalysis to localize the glacial refuge areas of *C. auronitens*

Dataset	Sample no. in this study	No. of samples	Loci (EC number)	No. alleles found	Sources (sample numbers in this study)
A	1–61	61	AAT (EC 2.6.1.1)	8	Assmann et al. (1994) (30–61); Assmann and Weber (1997) (1–29)
			EST (EC 3.1.1.1)	17	
			GPI (EC 5.3.1.9)	19	
			6PGDH (EC 1.1.1.44)	7	
			ME <sup>1</sup> (EC 1.1.1.40)	2 <sup>a</sup>	
B	30–77	48	AAT	4	Assmann et al. (1994) (30–61); Reimann et al. (2002) (62–77)
			EST	8	
			GPI	15	
			6PGDH	3	

<sup>a</sup>Data for polymorphisms at the ME locus partly unpublished: Samples 1–29 are monomorphic for one allele, samples 30–61 monomorphic for another allele



**Fig. 2** Sample sites covering the Western part of the distribution area of *C. auronitens*. Renumbered after Assmann et al. (1994), Assmann and Weber (1997) and Reimann et al. (2002), see Table 1

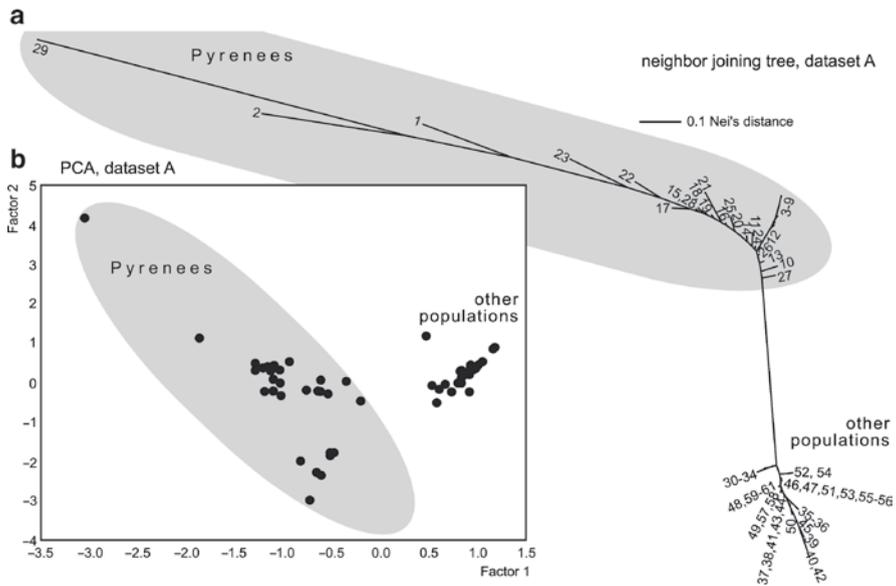
information on a total of 1,771 individuals from 61 sample sites in the Western and Southwestern part of the distribution range of *C. auronitens* (including the Pyrenees) at five loci, whereas dataset B contains information from 48 samples with a total of 1,517 individuals at four loci. Dataset B comprises additional samples, mainly from Western France but lacks the samples from the Pyrenees used in dataset A. Sample sites were renumbered and are shown in Fig. 2.

### 3.2.2 Statistical Analyses

Nei's standard genetic distances (Nei 1978) were calculated from allele frequencies and phenograms (using neighbor-joining, Saitou and Nei 1987) were constructed (with PHYLIP, Felsenstein 2005) for both datasets. Principal component analyses (PCA) based on the arc-sin transformed allele frequencies were performed for both datasets using STATISTICA, version 7.1 (StatSoft Inc.). For the latter analyses rare alleles were omitted, thus only alleles with a minimum mean frequency of 10% were used. Private alleles (defined as alleles found in one or two neighboring sample sites only) were counted per sample.

### 3.3 Genetic Differentiation and Localization of Glacial Refuges

The analysis of the allele frequencies of 61 samples of dataset A by both cluster analysis and PCA reveals concordant patterns. Whereas the samples from the Pyrenees – a comparably small geographic range – exhibit a striking genetic structure, partly with large genetic distances, the remaining populations sampled in a large part of the distribution area of *C. auronitens* show comparably small genetic differentiation (Fig. 3).

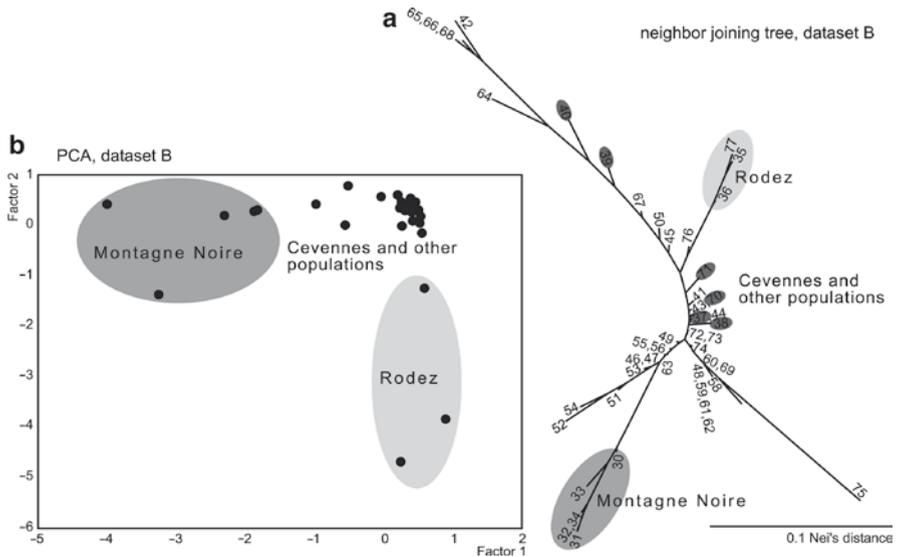


**Fig. 3** (a) Neighbor-joining dendrogram based on the genetic distances (Nei 1978) of 61 populations of *C. auronitens* (dataset A). For population numbers, see Fig. 2. (b) Principal component analysis of *C. auronitens* using dataset A. Populations from the Pyrenees are separated from the other populations on the first two factors, which explain 40.5% of the total variance (factor 1: 27.5%, eigenvalue 7.98; factor 2: 13.0%, eigenvalue 3.77)

Moreover, the latter samples are clearly separated from those from the Pyrenees. A special case provides sample no. 29 from the easternmost part of the Pyrenees (Mt. Canigou). Whereas Assmann and Weber (1997) could not decide to which of the two groups (Pyrenees or non-Pyrenees) this sample clustered, we find evidence for a similarity to the Pyrenean *C. auronitens* populations but with a relatively strong genetic distance.

The analysis of dataset B (excluding the populations from the Pyrenees) offers more detailed information. Despite a generally lower genetic differentiation, three groups can be distinguished by means of PCA that can also be found in the tree: the samples originating from the disjunct areas of the Montagne Noire and the region around Rodez form their own groups, whereas the other populations cluster together (Fig. 4). Populations originating from sample sites that are localized South of the Holdhaus line are highlighted in Fig. 4a to give an impression of possible glacial refuge areas. In contrast to samples from Rodez and Montagne Noire, populations from the Cevennes (nos. 37–40) and populations from the Southern part of the Auvergne (nos. 70–71) do not cluster together but can be found – from a genetic point of view – in the midst of the samples from all over the Western part of the large distribution range of the study species.

The results support the hypothesis of multiple glacial refuge populations of *C. auronitens* (already given by Assmann et al. 1994), most of which behaved



**Fig. 4** (a) Neighbor-joining dendrogram based on the genetic distances (Nei 1978) of 48 populations of *C. auronitens* (dataset B). For population numbers, see Figure 2. (b) Principal component analysis of *C. auronitens* using dataset B. Populations from Montagne Noire and Rodez are separated from the other populations on the first two factors, which explain 42.0% of the total variance (factor 1: 23.0%, eigenvalue 2.98; factor 2: 19.0 %, eigenvalue 2.47)



**Fig. 5** Glacial refuge areas of *C. auronitens*. Arrows show postglacial recolonization from two of these refuges, “?” indicates a putative additional refuge area or unknown migration routes (see text). The black line indicates the Holdhaus line

endemically or showed only restricted dispersal (i.e., in the Pyrenees or the Montagne Noire), whereas the populations from the Cevennes and the Southern Auvergne recolonized large parts of Western and Central Europe (Fig. 5). The strong power of dispersal of the populations at the edge of the distribution range is demonstrated by a recent recolonization event of *C. auronitens* populations in Northwestern Germany after anthropogenic landscape changes (Drees et al. 2008).

### 3.4 *Private Alleles: Evidence for a Refuge Area North of the Holdhaus Line*

Private alleles are, from a population genetic point of view, of special interest. They developed either during the course of a long-lasting isolation of the respective population (i.e., the variant originates from mutation) or they originally occurred in many populations but were lost at all but one site, e.g., as a consequence of repeated extinction and recolonization (Hewitt 1996). These two sources of origin imply that private alleles occur predominantly in populations in or close to refuge areas.

**Fig. 6** Numbers of private alleles in *C. auronitens* samples investigated at four allozyme loci. Groups of samples are indicated by a single circle only (compare Fig. 2), private alleles for these groups are summed over the respective populations



Altogether 24 private alleles were found in the *C. auronitens* populations, 14 in the populations from the Pyrenees and another three in the remaining samples from Southern France (Fig. 6). Additionally, seven private alleles occur in the region around the border triangle of Switzerland, France, and Germany (including the Vosges, the Black Forest, and part of the Swiss and French Jura). These findings suggest the existence of at least one other glacial refuge area in this region North of the Holdhaus line.

### 3.5 *Allele Numbers in Populations North and South of the Holdhaus Line*

Altogether 50 different alleles were recorded in populations situated South of the Holdhaus line, whereas only 23 different alleles were found in populations North of it. This is also reflected by the allele numbers corrected for the number of investigated populations (rarefied to 34 populations, Krebs 1999): The populations situated South of the Holdhaus line show 41.5 alleles, whereas the populations North of it have only 22.0 different alleles.

With the exception of the seven private alleles in the Black Forest, the Vosges, and parts of the Swiss and French Jura (see above), all alleles that were found in populations North of the Holdhaus line are also found in populations located South of it. The Northern populations are, therefore, a section of the genetic richness of the Southern populations.

## 4 Discussion

Although the Holdhaus line was introduced more than a half century ago, it is still up-to-date. The brief overview of the most recent literature on European cave beetles (including the comprehensive faunistic investigations in Northern Italy and Southern France) shows that only a few amendments, but no basic corrections, had to be made to the Holdhaus line.

Of what importance is the Holdhaus line for the identification of refugial areas for other endemic taxa? Recent studies of endemic-rich taxa indicate that the Holdhaus line is not only a remarkable Northern border for the distribution of blind cave- or soil-dwelling beetles, but is also congruent with the Northern distribution limit of several endemics (e.g., from the genus *Leistus*, Assmann and Heine 1993).

If highly specialized endogeic species were able to survive the glacial period(s) in Southern France and Northern Italy, i.e., in regions close to the Holdhaus line, it is likely that other species which are currently more widely distributed in Europe would also have had a survival chance in these “Massifs de Refuge.” Molecular analyses of populations of the ground beetle genus *Carabus* are a suitable method to localize former refuge areas, as shown for *Carabus solieri*, an endemic ground beetle species with a small postglacially recolonized area and a hybrid zone formed after a secondary contact of individuals from different glacial refuge areas (Garnier et al. 2004).

In our test species, *C. auronitens*, we found genetically rich but strongly diverging populations – rear edge populations according to Hampe and Petit (2005) and therefore of special importance for nature conservation. Moreover, these populations are obviously still situated close to their assumed glacial refuges South of the Holdhaus line (Fig. 5). Our analysis confirms the earlier ideas of multiple glacial refuges of *C. auronitens* in the Pyrenees, the Montagne Noire, close to Rodez, and in the Cevennes (Assmann et al. 1994; Assmann and Weber 1997; Reimann et al. 2002). Some of these refuge areas coincide with the putative refuges of the beech (*Fagus sylvatica*), as shown by a combination of palaeobotanical and genetic data (Magri et al. 2006). This example clearly illustrates the importance of the Holdhaus line in separating populations living in former glacial refuges from populations inhabiting postglacially recolonized areas. The Holdhaus line may also help to localize glacial refuge areas of other species, such as the fire salamander (*Salamandra salamandra*, Steinfartz et al. 2000), which prefers cold and humid conditions, and which recolonized parts of Europe postglacially.

The results of the genetic differentiation of *C. auronitens* do not mirror the sub-specific taxonomy of *C. auronitens* as there are at least two subspecies described (*C. a. auronitens* and *C. a. festivus*, Turin et al. 2003) which are not separated genetically. The morphometric analysis by Terlutter (1991) does not support this taxonomic system either. For *C. auronitens*, there are numerous taxonomic works in which several subspecies are described (e.g., Breuning 1932; Deuve 1994) which are partly contradictory and seem to lack any objective criteria. Thus, we strongly recommend renouncing the use of taxonomic units below species level for *C. auronitens* (for a general discussion, see Assmann et al. 2008).

Our results show that populations that are situated North of the line are genetically poorer than those located South of it. These populations display – from a genetic point of view – only a section of the refuge populations (with the exception of the populations from the French-German-Swiss border triangle, see below), which is in concordance with Reinig's (1938) idea of stepwise allele elimination in the course of a set of postglacial expansion processes.

As an exception, we even found populations in the Southwestern part of Central Europe (Jura, Vosges, and Black Forest) which contain private alleles. It seems improbable that these alleles originate from one of the putative refuge populations in Southern France as the populations situated in between these areas are genetically impoverished and display only a section of the allelic richness of the populations from the Cevennes. Surprisingly, a set of endemic taxa was described for this Southwestern part of Central Europe just after Holdhaus had published his compendium. It is noteworthy that some of these species share the same habitat with *C. auronitens*: five diplopod species (Spelda 1991), one carabid species (Huber and Molenda 2004), and *Lumbricus badensis* (Kobel-Lamparski and Lamparski 2004). All these species prefer humid and cold habitats. Furthermore, the Black Forest region is explicitly named as a potential glacial refuge area for the vole *Microtus arvalis* (Jaarola and Searle 2002).

Normally, there is a striking connection between blind beetles and certain bedrock or soil types. Although limestone bedrocks are not colonized exclusively, there is a main occurrence of blind beetles in these soils. Therefore, the lack of endogeic or troglobitic beetles in den Black forest region point to a certain shortcoming of the Holdhaus line.

Refuge areas on non-limestone bedrocks can thus be overlooked due to the lack of this special group of indicator species. It seems, then, that the limits demarcated by the Holdhaus line are further South than the possible geographic position of some glacial refuges for species of cold and humid habitats. Holdhaus seems to have recognized part of this problem as he explicitly named the records of blind troglobitic beetles in the Jura which caused him to draw an isolated enclave North of the continuous Northern distribution limit of blind troglobitic beetles. The putative glacial refuge area in the Black Forest is situated close to this enclave.

The data for *C. auronitens* as well as for many other species of cold-humid habitats (review by Schmitt 2007) further support Holdhaus' idea (Holdhaus 1906, 1954) that glacial refuge areas are located in the extra-Mediterranean. These results are in line with the findings of Willis et al. (2000) and Willis and van Andel (2004) who provide evidence for the existence of trees in many parts of Central Europe throughout the cold stages of the full and late glacial.

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# Postglacial Recolonization of Continental Europe by the Pygmy Shrew (*Sorex minutus*) Inferred From Mitochondrial and Y Chromosomal DNA Sequences

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**Abstract** The postglacial recolonization of the pygmy shrew (*Sorex minutus*,  $n=68$ ) across continental Europe was examined using control region sequences (342 bp) and concatenated sequences (2,939 bp) of four paternally inherited introns, specific for the Y chromosome. Phylogenetic analysis (NJ, MP, ML, and Bayesian inference) of the control region retrieved five major lineages (Northern, Western, Italian, Balkan, and Spanish) in Europe, in agreement with the previous cytochrome *b* data. Our study identifies a new refugium for the pygmy shrew located in France during the Pleistocene glacial cycles and the most important refugium with regards to the recolonization of Eurasia was probably located in the Carpathians. In contrast, Y chromosome intron sequences retrieved four major lineages (Northern, Western, Italian, and Balkan). This may indicate contact between Western and Spanish mtDNA lineages after expansion from their respective refugia post-LGM. Our results also revealed differential introgression of mtDNA and Y chromosome markers at a local level when populations from different glacial refugia come into contact. These results highlight the importance of adopting a multiple marker approach when inferring phylogeographic structure and colonization history of species.

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## 1 Introduction

The Pleistocene glaciations and intervening warming periods have undoubtedly played a major role in the formation of distinct genetic lineages across the geographic distributions of species (Hewitt 2000). The Last Glacial Maximum (LGM) occurred approximately from 23,000 to 19,000 calendar years before present (y BP; Mix et al. 2001). During this period, extensive areas of Europe were covered by ice and permafrost. Many species could only survive in relatively sheltered areas and would, therefore, have retreated to refugia with more suitable environmental conditions. Phylogeographic studies of European mammals have revealed several recolonization patterns from these refugia during the Pleistocene climatic fluctuations (Taberlet et al. 1998; Hewitt 1999). Glacial refugia that have been identified from a wide variety of studies of mammalian fauna include the Iberian Peninsula, Italy, and the Balkans (see Seddon et al. 2001; Taberlet et al. 1998; Mascheretti et al. 2003; Michaux et al. 2003). However the Mediterranean regions would have been more important as hotspots for endemism rather than a source of Northward recolonization for the rest of Europe (Bilton et al. 1998). The importance of refugia other than the Mediterranean peninsulas has become apparent through studies of a variety of mammals (Bilton et al. 1998; Stewart and Lister 2001). These include the Ural (Jaarola and Searle 2002; Brunhoff et al. 2003; Deffontaine et al. 2005) and Carpathian (Pazonyi 2004; Kotlík et al. 2006; Sommer and Nadachowski 2006; Saarma et al. 2007) mountain ranges. Some species of small mammals (e.g., bank vole *Myodes glareolus*) tolerated the climate conditions present during the LGM (Kotlík et al. 2006) to survive and recolonize Europe from these more Northern latitudes. Glacial refugia may also have been present further North in Scandinavia. For instance, populations of root voles, *Microtus oeconomus*, may have persisted through the LGM as far North as Andøya, an island situated off Northern Norway (Brunhoff et al. 2006).

Since the conception of phylogeographical studies, the most popular molecular markers have been mitochondrial DNA (mtDNA) sequences (Avise 2000). There are many reasons for this; mtDNA has many favorable properties, such as haploidy, a lack of recombination, rapid evolution compared to nuclear DNA (Brown et al. 1979), and is predominantly maternally-inherited (Nishimura et al. 2006). But perhaps the main reason for its widespread application is its relative ease of use (Petit et al. 2002) since universal primers were developed to amplify across a wide range of mammalian families (Kocher et al. 1989; Irwin et al. 1991). Despite the many obvious benefits, the sole use of mtDNA data can potentially lead to the construction of an incorrect species history because only the maternally-inherited mitochondrial lineage is being investigated (Ballard and Whitlock 2004). This is particularly important when it is evident that males and females do not play ‘symmetrical roles’ in establishing and maintaining population structure (Greenwood 1980).

Recently, the human genome sequencing project provided Y-specific genetic markers for humans (Petit et al. 2002). This has allowed numerous studies on the colonization history of humans (Underhill et al. 2000; Hammer et al. 2003) based

on male-inherited Y chromosome genes and the results have been consistent with the ‘Out of Africa’ hypothesis of modern humans based on mitochondrial, X-linked, and autosomal regions of DNA (Templeton 2002). However, there have been few phylogeographic studies in non-human mammalian species using Y chromosome markers. This may be due to the low levels of nucleotide diversity found in Y chromosome regions (Hellborg and Ellegren 2004). Recently, there have been studies on hybrid zones using Y chromosome structure (Jaarola et al. 1997) and Y-linked microsatellites (Balloux et al. 2000). Furthermore, combined datasets of mitochondrial, X and Y chromosomal genes have detected ‘cryptic species’ in the African elephant, *Loxodonta africana* (Roca et al. 2005), field vole, *Microtus agrestis* (Hellborg et al. 2005), and ground squirrels, *Spermophilus* (Gündüz et al. 2007). Brändli et al. (2005) used a similar set of markers to clarify the evolutionary history of the greater white-toothed shrew, *Crocidura russula* and its subspecies. Most of these studies have shown concordant tree topologies of mitochondrial, X and Y chromosomal DNA sequences when inferring evolutionary history, at the species- or subspecies-level. Therefore, the main finding of previous studies has been the reproductive isolation of genetic lineages. Little attention has been focused on sex-biased gene admixture when inferring the colonization history of a particular species.

The purpose of the present study is to investigate the female- and male-biased historical gene flow of the pygmy shrew, *Sorex minutus*. This study utilized a female-only inherited sequence (segment of the mitochondrial control region) and male-inherited sequences (four Y chromosome introns), and was centred on continental Europe and integrated in the context of the postglacial colonization history of the species. The pygmy shrew is the most widely distributed shrew species found in Europe (Mitchell-Jones et al. 1999) and it is also the oldest, with a fossil record dating back to the Pliocene, ca. 2 million years ago (Rzebik-Kowalska 1998). Its range extends from Western Iberia and Ireland to Siberia in the East, but is absent from Southern Iberia and most of the Mediterranean coasts and islands (Mitchell-Jones et al. 1999).

The pygmy shrew is an ideal choice for such a study because of its widespread distribution and fossil evidence of possible pygmy shrew survival in two refugia; with dates of approximately 17,000 y BP in Northern Spain (Pokines 2000) and 12–15,000 y BP in the Carpathian Basin (Pazonyi 2004). The pygmy shrew has also been the subject of previous postglacial colonization studies. Bilton et al. (1998) sequenced a 447 basepair (bp) segment of the cytochrome *b* gene from 14 specimens to reveal the importance of an unidentified glacial refugia located further North than the classic Mediterranean refugia. A similar result was found by Mascheretti et al. (2003) using a much larger dataset and a near-complete sequence (1,110 bp) of the cytochrome *b* gene when investigating the colonization history of Ireland. Glacial refugia identified thus far are the Iberian Peninsula, Italy, the Balkans, and a refugium located further North. Here we extend these findings using a combination of sex-linked and mitochondrial markers in an attempt to provide a more complete understanding of the pygmy shrew’s colonization history.

## 2 Materials and Methods

### 2.1 Sampling and DNA Extraction

A total of 68 samples were analyzed in this study. Forty eight of these samples were obtained from collections in the Universities of York, United Kingdom, and Lausanne, Switzerland. Live pygmy shrews were also sampled in France ( $n = 5$ ), including the island of Belle Île ( $n = 10$ ), using Trip Traps (Procter Bros. Ltd.). Details of sample locations are given in Table 1. DNA was extracted from ethanol-preserved tissue from tail tips, toe clippings or liver using the DNeasy Blood & Tissue Kit (Qiagen Ltd.) according to the manufacturer's protocol.

### 2.2 Mitochondrial DNA

The left domain of the control region was amplified using primers tRNA<sup>Pro</sup> (Stewart and Baker 1994a) and PS-CD (5' - GGCGAGATTAAATGTTAGCTGG - 3'). PCR reactions consisted of 2  $\mu$ l of DNA (40–200 ng/ $\mu$ l), 1.5  $\mu$ l (0.375  $\mu$ M; Sigma Aldrich) of forward and reverse primer and 45  $\mu$ l of MegaMix~Blue (Microzone Ltd.). PCR amplification was carried out in a Peltier Thermal Cycler (MJ Research) using an initial denaturation of 95°C for 3 min followed by 30 cycles of 95°C for 45 s, annealing temperature of 60°C for 1 min, and an extension of 72°C for 90 s. This was followed by a final extension step of 10 min. Fragment sizes ranged from approximately 500–800 bp, depending on the number of tandem repeats as previously reported in other *Sorex* species (Stewart and Baker 1994a; Fumagalli et al. 1996).

### 2.3 Y Chromosome Introns

Five primer sets were chosen from Y chromosome introns which amplified in the common shrew, *Sorex araneus* (Hellborg and Ellegren 2003). The introns chosen were DBY1, DBY3, DBY7, UTY11, and ZFY4 generating products totaling approximately 3.2 kbp. Two other introns (DBY8, DBY14), which amplified in *S. araneus* (Hellborg and Ellegren 2003) were not chosen due to a lack of polymorphism in several races of the common shrew (G. Yannic, unpublished data). Amplification of the Y chromosome introns was based on the protocol used by Hellborg and Ellegren (2003). Briefly, PCR reactions consisted of 3  $\mu$ l DNA (60–300 ng/ $\mu$ l), 5.0  $\mu$ l 10 $\times$ Buffer (Invitrogen), 2.5  $\mu$ l of MgCl<sub>2</sub> (2.5 mM; Invitrogen), 1.0  $\mu$ l dNTPs (0.2 mM; Invitrogen), 1.0  $\mu$ l (0.25  $\mu$ M; Sigma Aldrich) of forward and reverse primer for each locus and 0.3  $\mu$ l of Platinum Taq Polymerase (5 U/ $\mu$ l; Invitrogen) to make up a 50  $\mu$ l reaction.

PCR amplification was carried out using an initial denaturation at 95°C for 10 min followed by 20 cycles of 95°C for 30 s, a touchdown annealing temperature

**Table 1** List of samples and haplotypes (both mtDNA and concatenated Y chromosome introns) present in each locality. The number of individuals per locality is represented by *n*. The coordinates of each sampling locality (numbered from 1 to 38) is plotted in Figs. 3 and 4

Location	Country	Coordinates	Map		mtDNA	
			No.	<i>n</i>	haplotype	Y haplotype
Rascafria	Spain	40°54' N 03°53' W	1	3	Spa1,2	Spa1,2
Encamp	Andorra	42°35' N 01°35' E	2	2	And1,2	Fra3
Le Mas-d' Artige	France	45°42' N 02°08' E	3	1	Fra1	–
Nexon	France	45°45' N 01°15' E	4	1	Fra2	Fra1
Belle Île	France	47°20' N 03°11' W	5	10	BI1,2,3,4,5	BI1
Fresseneville	France	50°05' N 01°34' E	6	1	Fra3	–
Broualan	France	48°28' N 01°37' W	7	2	Fra4,5	Fra2,3
Plancoet	France	48°30' N 02°15' W	8	1	Fra6	Fra4
Morlaix	France	48°35' N 03°50' W	9	2	Fra6	Fra3
Boxtel	Belgium	51°36' N 05°20' E	10	1	Bel1	–
Wageningen	Holland	51°58' N 05°40' E	11	1	Hol1	–
Hartz Mountains	Germany	51°45' N 10°40' E	12	2	Ger1	Ger1,2
Eberswalde	Germany	52°10' N 13°45' E	13	1	Ger2	Ger3
Kobbero	Denmark	56°43' N 08°20' E	14	1	Den1	North1
Askland	Norway	58°40' N 08°35' E	15	3	Nor1,2,3	Nor1
Klockkarvik	Norway	60°07' N 05°00' E	16	2	Nor4	–
Steinkjer	Norway	64°00' N 13°53' E	17	1	Nor5	North1
Tromso	Norway	68°50' N 19°00' E	18	1	–	North1
Karlskrona	Sweden	56°09' N 15°15' E	19	1	Swe1	Swe1
Vehkalahti	Finland	60°15' N 27°00' E	20	1	Fin1	–
Val d' Illiez	Switzerland	46°16' N 06°43' E	21	1	Swz1	Swz1
Bretolet	Switzerland	46°13' N 06°40' E	22	1	Swz2	Swz1
Bassin	Switzerland	46°32' N 06°39' E	23	1	Swz3	–
Trento	Italy	46°15' N 11°50' E	24	2	Ita1,2	–
Abruzzo	Italy	42°00' N 14°00' E	25	4	Ita3,4,5,6	Ita1
Calabre	Italy	37°07' N 16°38' E	26	1	Ita7	Ita2
Reggio	Italy	44°32' N 11°21' E	27	1	Ita8	Ita3
Cesky Jiretin	Czech Republic	50°41' N 13°34' E	28	2	Cze1,2	North1, Cze1
Donnerskirchen	Austria	47°56' N 16°40' E	29	1	Aut1	Aut1
Bratislava	Slovakia	48°07' N 17°00' E	30	5	Svk1,2,3,4	North1, Svk1
Blizocin	Poland	51°36' N 22°16' E	31	2	Pol1,Pol2	North1,Pol1
Vilnius	Lithuania	54°40' N 25°19' E	32	2	Lit1	North1
Kanev	Ukraine	49°41' N 32°00' E	33	1	Ukr1	North1
Brjansk	Russia	52°20' N 34°00' E	34	1	–	North1
Novosibirsk	Siberia	54°49' N 83°06' E	35	1	Sib1	North1
Lake Baikal	Siberia	53°40' N 108°00' E	36	1	–	Sib1
Pelister Mountain	Macedonia	41°00' N 21°10' E	37	1	Mac1	–
Strandzha Mountains	Turkey	41°45' N 27°41' E	38	2	Tur1,2	Tur1

from either 60–50°C (DBY1, DBY7) or 55–45°C (DBY3, UTY11) for 1 min, decreasing by 0.5°C per cycle and then an extension of 72°C for 90 s. This was followed by 20 cycles of 95°C for 30 s, 50°C (DBY1, DBY7) or 45°C (DBY3, UTY11) for 1 min, and 72°C for 90 s and a final extension step at 72°C for 10 min. All PCR reactions included a known female sample as a negative control. Amplification of a fragment in the female sample was used as evidence that the gene fragment was not specific to the Y chromosome. For this reason, intron ZFY4 was disregarded after amplification in several female samples.

## 2.4 Sequencing

PCR products were purified using Novagen® Spinprep™ PCR Clean-up Kit (Merck Biosciences) according to the manufacturer's protocol. Control region fragments were sequenced using the forward (tRNA<sup>Pro</sup>) and reverse primers (PS-CD). Y chromosome introns, DBY3 and DBY7, were sequenced using forward primers only while introns, DBY1 and UTY11, were sequenced using forward and reverse primers. Sequencing was carried out by Macrogen Inc. (Seoul, Korea). Control region fragment and Y chromosomal intron sequences were deposited in GenBank (Accession nos. for control region fragments: EU564340–EU564393; DBY1 intron: EU564394–EU564407 and EF636561–EF636562; DBY3 intron: EU564408–EU564414 and EF636573; DBY7 intron: EU564415–EU564422 and EF636584; UTY11 intron: EU564423–EU564434 and EF636606. Concatenated Y chromosomal intron sequences are available upon request).

## 2.5 Data Analysis

Chromatogram contiguous sequences were assembled in Sequencer 4.5 (GeneCodes) and sequences were aligned manually using Se-Al 2.0 (Rambaut 1996). The 5' end of the *Sorex* control region contains a tandem repeat region (Stewart and Baker 1994a,b; Fumagalli et al. 1996), with repeat motifs ranging from 78 to 80 bp in length. The number of tandem repeat copies ranged from 3 to 7 per individual and length heteroplasmy due to repeat numbers within and between individuals was present. All tandem repeat copies within an individual were screened for mutations between repeats (Rhymer et al. 2004). For analyses, in accordance with previous phylogenetic studies on *Sorex* species (Stewart and Baker 1994a,b, 1997; Rhymer et al. 2004) only the last tandem repeat and the unique flanking regions at the 5' end containing the hypervariable region were used, in addition to the 3' end of the repeat region (including part of the conserved Central Domain). This resulted in 342 bp (including indels) being used in the analyses. Chromatogram contigs for the four Y introns were assembled and

analyzed individually before being combined in Se-AL 2.0 (Rambaut 1996) to form a concatenated dataset (2,939 bp including indels).

Control region and concatenated Y intron haplotypes were identified using DAMBE 4.5.50 (Xia and Xie 2001). Nucleotide diversity ( $\pi$ ) was calculated using DnaSp 4.10.9 (Rozas et al. 2003). Net divergence ( $Da$ ) was calculated in MEGA v. 3.1 (Kumar et al. 2004) using Kimura's 2-parameter distances (Kimura 1980). Standard errors (SE) were calculated by bootstrapping with 10,000 replicates.

Neighbor-joining (NJ), Maximum Parsimony (MP) and Maximum Likelihood (ML) phylogenetic trees derived from control region haplotypes were constructed in PAUP\* 4.0b10 (Swofford 1998). ML and NJ trees were constructed using the HKY85+I+G (Hasegawa et al. 1985) model ( $I=0.39$ ;  $\alpha=0.7831$ ), selected by the Akaike Information Criterion (AIC) in ModelTest 3.06 (Posada and Crandall 1998). Indels were treated as missing data when constructing trees. Of the 342 bp that were sequenced, 72 characters were parsimony informative. Bootstrap support was calculated from 1,000 NJ, MP, and ML parametric replicates, respectively. Bayesian posterior probabilities were estimated in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) from 1,000,000 generations sampled every 1,000th generation excluding a burn-in of 100,000 steps. *Sorex volnuchini* was used as the outgroup species, in accordance with previous phylogeographic studies on *S. minutus* (Bilton et al. 1998; Mascheretti et al. 2003).

The amount of genetic variation among and within phylogenetic lineages was determined by an analysis of molecular variance (AMOVA) implemented in Arlequin version 3.11 (Excoffier et al. 2005).

Recent population expansion from small founder populations for control region data was tested using mismatch distributions and Fu's (1997)  $F_s$ . Mismatch distributions of pairwise nucleotide differences of each lineage were calculated and compared with expected values for an expanding population (Rogers and Harpending 1992). Tests for goodness-of-fit statistics based on the sum of square deviations (SSD) for a model of sudden expansion were determined in Arlequin version 3.11 (Excoffier et al. 2005). Fu's  $F_s$  tests were performed to detect an excess of low frequency mutations in each lineage conforming to a model of sudden expansion. Tests for goodness-of-fit and Fu's  $F_s$  were generated in Arlequin v. 3.11 using parametric bootstrapping with 10,000 replicates.

Because sequence regions of the control region evolve at different rates (Stewart and Baker 1994a; Pesole et al. 1999), expansion times for lineages conforming to the sudden population expansion model were estimated using the unique sequence region at the 5' end from the mismatch distribution ( $\tau$ ) of each lineage, using the formula  $\tau = 2 \mu t$  (where  $\tau$  is the time since expansion in units of  $1/2 \mu$  generations), using a generalized least squares approach adapted from Rogers (1995), as described in Schneider and Excoffier (1999). Generation time was assumed to be 1 year (Churchfield and Searle 2008). An average evolutionary rate of 23% (15–31%) per million years ( $\text{My}^{-1}$ ) was used based on the rate derived from other *Sorex* species for the unique sequence region at the 5' end (Stewart and Baker 1994a). The divergence time ( $T$ ) between lineages for control region data was estimated by  $T = Da/2\mu$ , where  $2\mu$  is the divergence rate of 23%  $\text{My}^{-1}$  for the unique sequence

region at the 5' end with 95% confidence intervals (CI) estimated according to Jaarola and Searle (2004).

A median-joining network of Y chromosomal introns was constructed using Network 4.2 (Bandelt et al. 1999) to illustrate relationships among haplotypes. Indels were included as informative characters (Hellborg et al. 2005). Bootstrap support for lineages was calculated separately in PAUP\* 4.0b10 from 1,000 NJ and MP replicates, respectively. NJ analysis was performed using Kimura's 2-parameter distances (Kimura 1980) due to differences in the substitution patterns of the four introns (Hellborg et al. 2005).

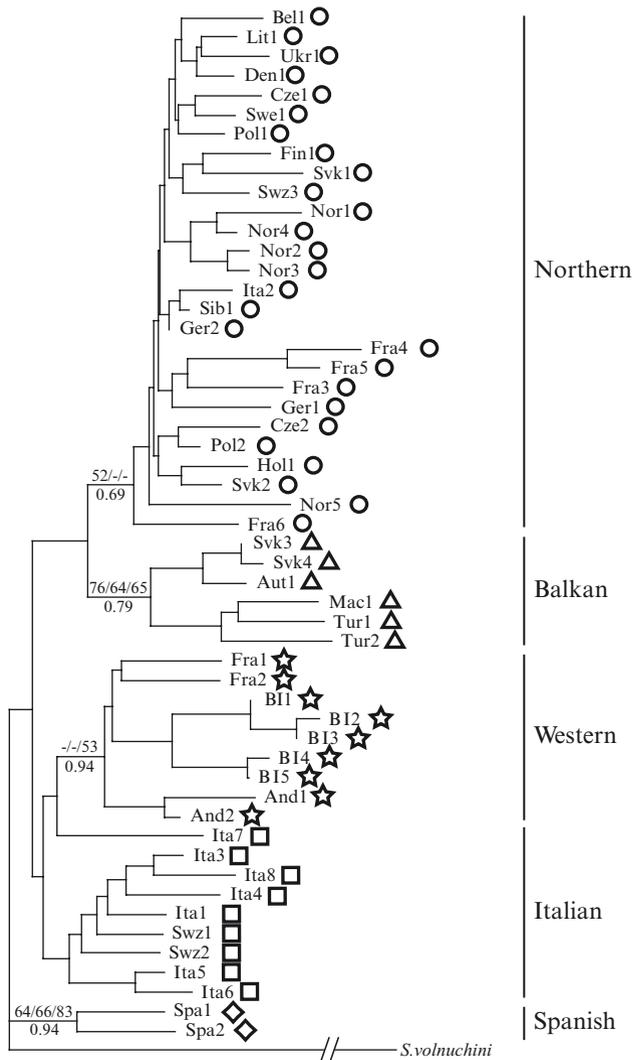
### 3 Results

#### 3.1 Mitochondrial DNA Data

We examined 63 pygmy shrews from continental Europe for 342 bp of the mitochondrial control region. Of the 342 nucleotides, 108 were polymorphic (including indels). Fifty one (47.2%) polymorphic sites were found in the 5' unique flanking region. Thirty (27.8%) polymorphic sites were found in the last tandem repeat which was 78–80 bp in size and 27 (25%) polymorphic sites were found in the 3' end and part of the Central Domain. Fifty three unique haplotypes were identified (see Table 1). Only one haplotype was shared between localities: Fra6 between two localities in Northern France. NJ, MP, ML, and Bayesian analysis of the 53 haplotypes revealed concordant topologies resulting in five major lineages (Fig. 1), with poor to relatively good statistical support. The Western lineage included individuals from Andorra, central France, and the French island Belle Île. The Northern lineage included individuals from Northern France and Norway in the West to Siberia in the East. The Spanish lineage was confined to Spain. The Balkan lineage was present as far North as Austria and Slovakia, and the Italian lineage was present in Italy and the Alps in Switzerland and France. One outlier haplotype, Ita7, was identified from this region (Fig. 1). The Northern lineage is separated from both Western and Italian lineages by two indels, 3 bp and 4 bp in size. The Spanish lineage is separated from the Northern lineage by an 8 bp indel, and the Spanish and Balkan lineages are separated by a 2 bp indel. This corroborates the lineages identified by Mascheretti et al. (2003) using cytochrome *b* but with certain lineages more widespread because of better sampling coverage. An AMOVA showed that the majority of control region variation (62.8%) was distributed among lineages with a little variation (10.13%) found among populations within lineages.

The nucleotide diversity ( $\pi$ ) for lineages ranged from 0.01786 to 0.0331 (Table 2). Net divergence ( $Da \pm SE\%$ ) between lineages and estimated divergence in 1,000 s of calendar years before present (ky BP) are given in Table 3. Mismatch distributions for four out of the five lineages significantly conformed to the sudden population expansion model (Table 4). The Spanish lineage was excluded because of the small sample size ( $n=3$ ). Fu's  $F_s$  values and estimates for time since expansion for the four lineages based on Rogers (1995)  $\tau$  are given in Table 4.

— 0.005 substitutions/site



**Fig. 1** Neighbour-joining tree using the HKY85+I+G model of substitution of 53 control region haplotypes (342 bp) showing the 5 lineages found in European *S. minutus*. See Table 1 for haplotype designations. Bootstrap values obtained by NJ, MP, ML (>50%) are shown above nodes and Bayesian probabilities (>0.50) are shown below nodes for each lineage

### 3.2 Y Chromosome Intron Data

We examined 39 pygmy shrews for 2,939 bp (including indels) of concatenated Y introns (DBY1: 1098 bp, DBY3: 596 bp, DBY7: 441 bp, UTY11: 804 bp), which resulted in 23 unique haplotypes (see Table 1 and Fig. 2). Sixty polymorphic sites

**Table 2** Nucleotide diversity ( $\pi \pm \text{SD}$ ) of and number of individuals ( $n$ ) in each mtDNA and concatenated Y chromosomal intron lineage

Lineage	$n$	mtDNA	Y chromosome introns	
		$\pi \pm \text{SD}$	$n$	$\pi \pm \text{SD}$
Northern	30	0.02706 $\pm$ 0.00176	23	0.00033 $\pm$ 0.00009
Western	14	0.03193 $\pm$ 0.00334	10	0.00084 $\pm$ 0.00017
Italian	9	0.02746 $\pm$ 0.00276	5	0.00136 $\pm$ 0.00048
Balkan	7	0.02863 $\pm$ 0.00516	1	–
Spanish	3	0.01786 $\pm$ 0.00842	–	–
–	–	–	–	–
Total	63	0.04406 $\pm$ 0.00181	39	0.00384 $\pm$ 0.00034

**Table 3** Estimates of percentage divergence ( $Da \pm 95\%$  CI) between control region lineages (upper diagonal) and estimates of divergence times [ky BP ( $\pm 95\%$  CI)] (lower diagonal) using an evolutionary rate of 23% My<sup>-1</sup>

ky/ $Da \pm \text{SE}\%$	Northern	Western	Italian	Balkan	Spanish
Northern	–	6.06 $\pm$ 2.13%	5.09 $\pm$ 1.97%	5.53 $\pm$ 2.21%	5.45 $\pm$ 2.14%
Western	263 (171–356)	–	3.08 $\pm$ 1.45%	8.22 $\pm$ 2.73%	3.62 $\pm$ 1.74%
Italian	221 (136–307)	134 (71–197)	–	6.93 $\pm$ 2.58%	3.03 $\pm$ 1.73%
Balkan	240 (144–337)	357 (239–476)	301 (189–413)	–	6.49 $\pm$ 2.26%
Spanish	237 (144–330)	157 (82–233)	132 (57–207)	282 (184–380)	–

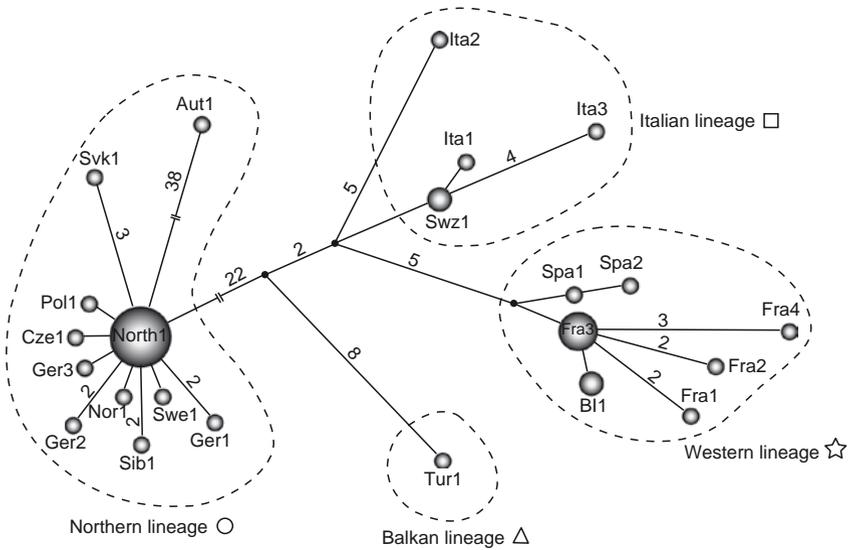
**Table 4** Estimates of SSD,  $F_s$ ,  $\tau$  and time since expansion (TSE) in y BP for each lineage

mtDNA lineage	SSD	$F_s$	$\tau$ (95% CI)	TSE (95% CI)
Northern	0.00375	–15.945*	4.088 (2.801–5.260)	72,844 (49,911–93,728)
Western	0.07546	0.680	4.578 (0.420–8.410)	87,300 (8,009–160,374)
Italian	0.00367	–2.661	3.363 (1.408–6.920)	64,698 (27,087–133,128)
Balkan	0.03880	0.083	3.234 (0.025–6.068)	54,499 (421–102,258)

\*Indicates significance ( $p < 0.05$ )

(including indels) were found in the concatenated Y chromosome dataset. Twenty-two (36.7%) polymorphic sites were found in DBY1; 8 (13.3%) in DBY3; 8 (13.3%) in DBY7, and 22 (36.7%) in UTY11. A 4 bp indel separated the Northern lineage from all other samples in DBY1 and the Northern lineage also possessed an extra repeat motif (CAAA) in the UTY11 intron.

Four major lineages were identified from the concatenated dataset by the median-joining network (Fig. 2). The Western Y lineage consisted of individuals from the Western and Spanish mtDNA lineages plus individuals from the Northern mtDNA lineage found in Northern France (Fig. 2). The Northern Y lineage included all individuals found in the Northern mtDNA lineage with the exception of those from Northern France and also included individuals from Austria and Slovakia, which belonged to the Balkan mtDNA lineage. The Italian Y lineage contained the same individuals as the Italian mtDNA lineage. Bootstrap support was high for



**Fig. 2** Median-joining network of 23 concatenated Y chromosomal intron haplotypes (2,939 bp). Numbers on branches indicate more than one mutation. Inferred haplotypes are represented by black dots. Bootstrap support for the Northern Y lineage is 100% and support for the Western Y lineage is 96% using both NJ and MP methods, respectively. See Table 1 for haplotype designations

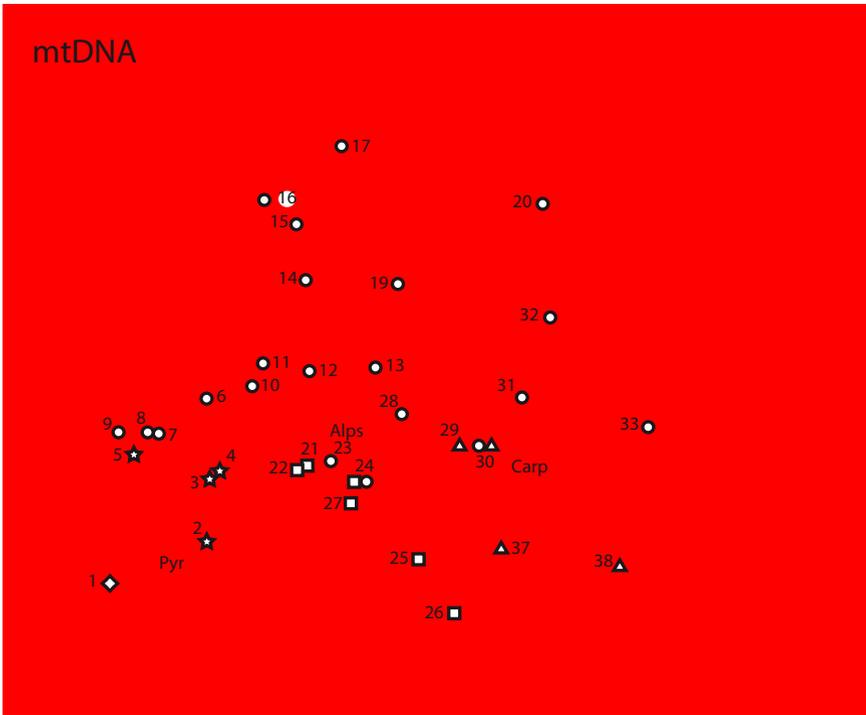
Western (NJ/MP: 96/96%) and Northern (NJ/MP: 100/100%) Y lineages, with weak support for the Italian Y lineage. Only one individual belonged to the Balkan Y lineage. These lineages were also identified from each Y intron individually, with the exception of DBY3 which failed to separate the Western and Italian lineages (data not shown). The nucleotide diversity ( $\pi$ ) of each lineage is given in Table 2.

Eleven haplotypes were found in 23 individuals in the Northern Y lineage (see Table 1 and Fig. 2), with Y haplotype North1 found in 10 localities spread from Norway to Siberia. The Y haplotype Aut1 had several large indels. Seven haplotypes were found in the Western Y lineage and four in the Italian Y lineage. However, the Y haplotype Ita2 was quite different from other Italian individuals (Fig. 2). This was also the case for this individual in the control region analysis (haplotype Ita7; Fig. 1).

## 4 Discussion

### 4.1 Postglacial Colonization History Inferred From mtDNA

The five lineages identified from control region data agrees with previously published data for the cytochrome *b* gene (Mascheretti et al. 2003). However, statistical support for all but two (Balkan and Spanish lineages) of these control region lineages were



**Fig. 3** Distribution of control region lineages of continental Europe (see Fig. 1 for symbol designation of lineages). Samples 34–36 belonging to the Northern mtDNA lineage are not shown. Clustered grey triangles represent the major mountain ranges; Pyr=Pyrenees and Carp=Carpathians

generally very low (<75%, Fig. 1), and this may be due to the large amount of variation and indels present in the *Sorex* control region. Although the Mediterranean peninsulas were important refugia for the pygmy shrew, they contributed relatively little to the spread of the pygmy shrew throughout Europe (Bilton et al. 1998).

From Fig. 3, the Spanish lineage appears to be confined to Spain which could indicate that the Pyrenees separated the glacial refugia for the Spanish lineage from that of the Western lineage in Southern France. The Andorran samples belong to the Western lineage as reported previously (Mascheretti et al. 2003) and could represent a Southern colonization after the LGM. Likewise, the Alps could have acted as a barrier between the Italian and other lineages at the LGM, with a subsequent spread of the Italian lineage into the mountains from an Italian refugium after the LGM.

With regards to the possible glacial refugia for the pygmy shrew Western lineage in Southern France, this is a new refugial location not previously suggested for the pygmy shrew. The common vole, *Microtus arvalis*, is proposed to have persisted North of the Pyrenees (Heckel et al. 2005) and fossil finds of the common shrew (*S. araneus*) or Millet's shrew (*S. coronatus*) indicate that it, along with other

temperate species, survived in the Dordogne in South-Western France during the LGM (Sommer and Nadachowski 2006). From such a refugium, the Western lineage could have easily spread further North to the small French island, Belle Île, which only became separated from the French mainland approximately 8,000 years ago (A. Tresset, pers. comm.). This accounts for the relatively high nucleotide diversity (data not shown) present on the island compared to large islands, such as Ireland (McDevitt et al. 2009) which was probably colonized by a small number of individuals introduced by humans (Mascheretti et al. 2003; McDevitt et al. 2009). The Balkan lineage is present as far North as Slovakia and Austria to which it presumably spread from a Balkan refugium. Five individuals from the same locality in Slovakia include individuals representing two separate lineages, the Northern and Balkan. This mtDNA contact zone is located near the Carpathians which is also likely to have been a glacial refugium during the LGM for the pygmy shrew (Pazonyi 2004) as well as other mammalian species (Kotlík et al. 2006; Saarma et al. 2007). We propose that it was the Northern lineage that occupied this refugium. However, further sampling with mtDNA is needed to provide more support for this and other refugial locations.

The most widespread mtDNA lineage of the pygmy shrew found in Europe is the Northern lineage which occurs from Northern France in the West to Siberia in the East to the Scandinavian countries in the North. Mismatch distribution and a negative and significant  $F_u$ 's  $F_s$  value indicate that this lineage originated from a single refugium and spread rapidly. The extensive distribution of this lineage may be due to a selective advantage of this mtDNA type in North temperate and arctic environments. Fontanillas et al. (2005) showed that certain mtDNA haplotypes in *C. russula* may be better adapted to colder environments than others, and this was particularly evident in females. A similar selective advantage has also been shown in humans (Mishmar et al. 2003) and been advocated in hares (Melo-Ferreira et al. 2005). Pygmy shrews inhabiting regions, such as the Carpathians during glacial cycles may have been better adapted to the temperature regimes found in North temperate and arctic regions than those present in refugia in the Mediterranean peninsulas and Southern France.

The approximate divergence times between the mtDNA lineages of the pygmy shrew indicate that separation may have occurred several glacial cycles ago (Renssen and Vandenberghe 2003). This is also the case with other small mammals in Europe (Jaarola and Searle 2004; Deffontaine et al. 2005). Larger, more mobile mammals show a lack of phylogeographical structuring pre-LGM (Hofreiter et al. 2004) but small mammals are more limited in their dispersal abilities and would, therefore, have remained isolated between glacial cycles, promoting divergence in mtDNA sequences. On the assumption of the molecular clock used, the Balkan and Northern lineages diverged from all other lineages approximately 240–357,000 and 221–263,000 y BP, respectively. The Western, Italian, and Spanish lineages diverged more recently (approximately 132–157,000 y BP).

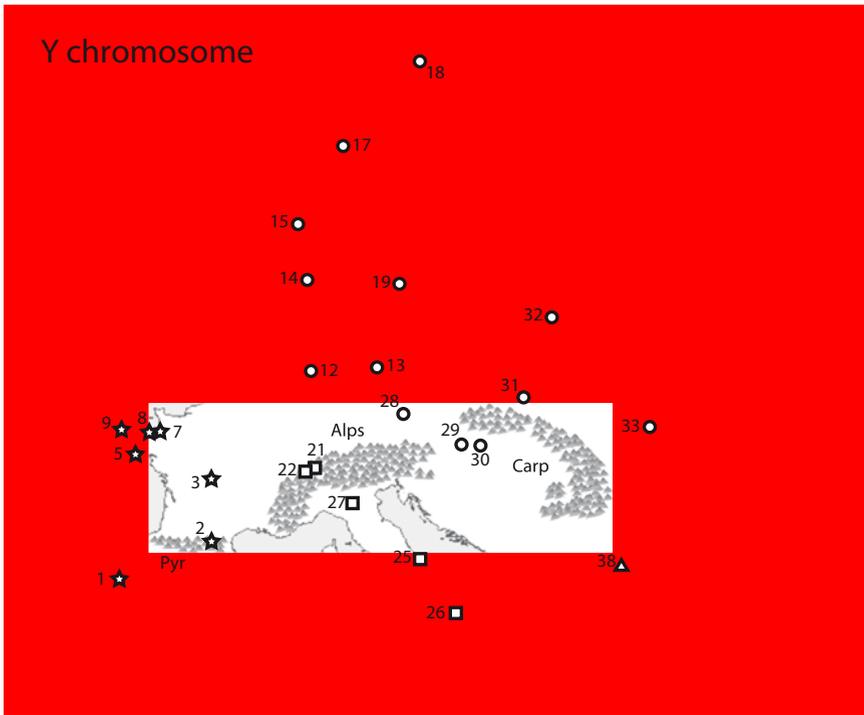
Estimates of time since population expansion vary among lineages. The Northern and Italian lineages expanded 72,844 and 64,498 y BP, respectively, with lower 95% CI values (49,911 and 27,087 y BP, respectively) before the LGM

(19–23,000 y BP). Estimates for the other two lineages were somewhat similar. The estimate for the Western lineage is 87,300 y BP with a lower 95% CI value (8,009 y BP) which stretches into the Holocene. This was affected by the large sample size from Belle Île as the estimate was lower when excluding individuals from the island (57,952 y BP; 95% CI: 11,575–108,009 y BP). The Balkan lineage has an estimate of 54,499 y BP, but with a large 95% CI range (421 years BP–102,258 y BP). These large 95% CI ranges (approximately 50–100,000 y) are obviously affected by the low sample size used for all lineages apart from the Northern lineage. It is also worth noting that only the Northern lineage had a negative and significant  $F_s$  value. The data here support the view that lineages might have expanded during or after the Early Pleniglacial (74–59,000 y BP; Renssen and Vandenberghe 2003) but for all except the Northern and Italian lineages a later date for the expansion, after the LGM, cannot be excluded.

However, the results presented here should be treated with caution as the evolutionary rate varies greatly among different regions of the *Sorex* control region (Stewart and Baker 1994a). A divergence rate of 15–20%  $\text{My}^{-1}$  has been used previously for *S. araneus* (Andersson et al. 2005) based on an error in the abstract of the original paper (D. Stewart, *pers. comm.*). The true divergence rate quoted in the discussion section of Stewart and Baker (1994a) is 8.3–14.3%  $\text{My}^{-1}$  for the unique sequence and repeat regions combined. The value used here (23%  $\text{My}^{-1}$ ) for the 5' end of the unique flanking sequence may in fact underestimate the divergence between lineages and their expansion times. Evolutionary rates as high as 60.4%  $\text{My}^{-1}$  for the control region have been reported in *M. arvalis* (Heckel et al. 2005). The use of this value places the expansion of lineages as follows; Northern: 27,738 y BP; Western: 33,243 y BP (22,067 y BP without individuals from Belle Île); Italian: 24,636 y BP, and Balkan: 20,753 y BP with lower 95% CI values occurring at the end or after the LGM (160 y BP–19,006 y BP). This interpretation fits much better with the expectation of small refugial populations at the LGM and extensive expansion thereafter.

## 4.2 Postglacial Colonization History Inferred From Y Chromosomal Introns

Four major Y chromosome lineages were identified throughout Europe as opposed to five identified using mtDNA (Fig. 2). One of the major differences between female-mediated (mtDNA) and male-mediated (Y introns) lineages was the strong grouping of all French and Spanish shrews to form a Western Y lineage (Fig. 4). Pygmy shrews from Northern France belong to the Western Y lineage as opposed to the Northern lineage for mtDNA. The well supported Western Y lineage is possibly caused by the slower mutation rate in Y chromosome genes compared to faster evolving mitochondrial genes (Hellborg and Ellegren 2004). According to the mtDNA data, the Western, Spanish, and Italian mtDNA lineages diverged more recently from each other than the Northern and Balkan mtDNA lineages (Table 3),



**Fig. 4** Distribution of concatenated Y chromosomal intron lineages of continental Europe (see Fig. 2 for symbol designation of lineages). Samples 34–36 belonging to the Northern Y lineage are not shown. Clustered grey triangles represent the major mountain ranges; Pyr=Pyrenees and Carp=Carpathians

and the grouping of French and Spanish Y haplotypes in the same Y chromosome lineage may reflect a more ancient association. However, it could be expected that French, Spanish, and Italian individuals would group together in a Y lineage if this was the case, and although the Italian Y lineage is poorly supported, it is clearly distinct from the Western Y lineage (Fig. 2). Therefore, it is possible that there was gene flow between Spanish and French refugia more recently than the mtDNA data suggests, perhaps after they came into contact after the range expansion following the LGM.

The Y chromosome data supports the existence of a separate glacial refugium in Italy for the pygmy shrew, as already suggested by the mtDNA data. Interestingly, the individual from Southern Italy (Y haplotype Ita2, mtDNA haplotype Ita7; Figs. 3 and 4) did not group with the other Italian haplotypes for both Y chromosome introns and mtDNA. There may have been multiple refugia within Italy during the Pleistocene glaciations, causing isolation of certain populations from others. Further samples are needed to fully resolve this. Genetic differentiation has been

reported within the Italian peninsula for *Sorex antinorii* because of putative post-glacial recolonization routes (Basset et al. 2006).

The Northern Y lineage is typified by low nucleotide diversity, in comparison to the other lineages (with the exception of the Balkan Y lineage where only one individual was sequenced). Only 11 haplotypes were found in 23 individuals (Fig. 3), with the North1 Y haplotype found in 13 individuals from 10 locations ranging from Norway in the Northwest to Siberia in the East. This supports the contention that expansion of the mtDNA Northern lineage over much of Northern Eurasia occurred from a single refugium. Individuals from the Balkan mtDNA lineage in Slovakia and Austria belonged to the Northern Y lineage. As is the case for Northern France, this indicates differential introgression of mtDNA and Y chromosome markers at a local level when populations from different glacial refugia come into contact.

## 5 Conclusions

Analyses of multiple molecular markers with different means of inheritance has demonstrated contrasting patterns of recolonization for the pygmy shrew in continental Europe. Five major lineages were retrieved from mtDNA data, in accordance with Mascheretti et al. (2003). In contrast, four major lineages were retrieved from Y chromosome data. By using mtDNA alone, contact between French and Spanish refugia would not have been identified and this study has highlighted the importance of adopting a multiple marker approach in phylogeographic studies.

The pygmy shrew is the oldest shrew found in Europe (based on fossil data; Rzebik-Kowalska 1998) and is relatively rare on the European mainland (often restricted to high altitudes) in comparison to other shrew species because of competition with species which occupy similar niches and as a result of habitat fragmentation. The genetic lineages described in this study diverged several glacial cycles ago and therefore each has an ancient evolutionary history (Jaarola and Searle 2004). Some of these lineages are relatively rare on the European continent in comparison to others. For example, the Northern lineage is spread throughout Northern and Eastern parts of Europe while the other lineages are relatively restricted in their distribution, often confined to the Southern refugial regions. Fully identifying refugial locations and protecting reservoirs of genetic diversity is vital for the long-term persistence and conservation of pygmy shrew populations (Bhagwat and Willis 2008).

**Acknowledgments** We thank Damien and Anne McDevitt, Jacqueline Fox, and Marine Pascal for field assistance in France. Thanks to Vitaly Volobouev and Michel Pascal for providing the appropriate licences for trapping and Alain Butet, Anne Tresset, and Carlos Nores for trapping advice. We particularly thank John O'Brien, James Carolan and everyone in Research Lab 4 (UCD) for technical assistance in the laboratory. We thank Don Stewart, John O'Brien, Rodrigo Vega, and Jerry Herman for their help and comments on the earlier drafts of the manuscript

and Olaya Astudillo for help with figures. A.D. McDevitt was funded by the Irish Research Council for Science, Engineering and Technology (IRCSET) and a travel grant to France awarded by Enterprise Ireland. G. Yannic was supported by a grant from the Fondation Agassiz (Switzerland). J.B. Searle received field work funding from the Arts and Humanities Research Council (UK).

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# Are Disjunct Alpine and Arctic-Alpine Animal and Plant Species in the Western Palearctic Really “Relics of a Cold Past”?

Thomas Schmitt, Christoph Muster, and Peter Schönswetter

**Abstract** The climatic fluctuations during the Pleistocene as well as the Holocene warming caused numerous disjunctions of cold-adapted, arctic-alpine, and alpine biota. However, the depths of the genetic splits among the disjunct parts of the species distributions vary considerably. The arctic ranges are usually weakly differentiated, and great similarity with at least some areas in more Southern regions is frequently found. Likewise, major mountain ranges in geographic proximity often share genetically similar populations. However, the genetic constitution of populations from more remote (predominantly Southern) mountain systems is strongly different from all other populations. This suggests recent vicariance events in the two former groups, but long-lasting isolation in the latter group, which apparently is mostly composed of relics of a more distant cold past.

## 1 Introduction

The world’s climate has undergone dramatic changes during the history of the planet. The most recent period of climatic instability, the Pleistocene, was characterized by particularly strong and more or less regular oscillations between warm and predominantly moist, and cold and more often dry periods (Williams et al. 1998). During the cold stages, thermophilic species (e.g., Mediterranean or temperate taxa)

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were restricted to Southern refugia in the Southern European peninsulas where they have survived in refugia and expanded their ranges during warm periods such as the interglacials or the postglacial (de Lattin 1967; Müller 1980; Hewitt 1996, 2000, 2004a,b; Taberlet et al. 1998; Schmitt 2007). For temperate species, this view has, however, been challenged by the recent combination of pollen data and genetic analyses (Steward and Lister 2001), suggesting that refugia of, e.g., the Common Beech (*Fagus sylvatica*), were situated much further North than previously suggested (Magri et al. 2006).

The cycles of area regression and expansion of species currently restricted to high mountain habitats and/or high latitudes were likely diametrical: these species were potentially widely distributed during cold stages and experienced range restrictions during warm periods (de Lattin 1967). Consequently, current distribution patterns of species adapted to high altitudes or high latitudes are often strongly disjunct and many of the isolated area fragments are small and widely separated from the core of their distribution area (Varga and Schmitt 2008).

The application of molecular techniques to reconstruct genealogical relationships above, at or even below the species level, has created a powerful tool to unravel the biogeographic history of taxa in space and time (Hewitt 1999, 2001, 2004a; Taberlet et al. 1998; Avise 2000; Schmitt 2007). We here review phylogeographic data on high mountain and high latitude species with disjunct distribution areas hitherto available for the Western Palearctic. In the following, we distinguish between two distributional types of species: (1) alpine, montane, and dinodal species of the European mountain system (EMS) of temperate regions sensu Ozenda (1988) and (2) arctic-alpine species. For each of these two above categories we summarize published studies and some unpublished results, and we search for congruencies as well as differences among the observed phylogeographic patterns. Based on the degree of genetic divergence between isolated “relic” taxa or populations and those from the main distribution areas, we aim to unravel if these entities represent relics of a cold past or are rather descendants of fairly young range expansions.

## **2 Species of the European Mountain System (EMS) of Temperate Regions**

The distributions of species of the EMS as defined here include the Cordillera Cantábrica and range from there over the Pyrenees, Alps, and Apennines to the Carpathians and the mountains of the Northern and central Balkan Peninsula. They normally do not occur South of the Pyrenees or of the Italian Abruzzo, and are also lacking from the mountain ranges of the Southern Balkan Peninsula South of the Adamović line (Ozenda 1988). Few species of this category occur throughout the entire outlined area. While some of these mountain species are restricted to a part of the EMS where they are fairly frequent, others exhibit much wider but scattered distribution areas.

## 2.1 *Alpine Species*

During the last few years, an ample body of literature has accumulated exploring the phylogeography of alpine plants of the EMS. A review of hitherto available studies on Alpine plants was presented by Schönswetter et al. (2005). Therefore, we here consider only studies on species that show disjunctions among mountain ranges. For this group of alpine species, it could be shown by various examples in plants and animals that (1) the Alps often own more than one genetic lineage, (2) mountain areas in geographic proximity to the Alps may share genetic lineages with the adjacent regions of the Alps, and (3) more remote mountain areas mostly show strongly differentiated genetic lineages underpinning long geographic separation of these remote mountain areas and old relic status of their populations.

In the Alpine-Pyrenean disjunct *Phyteuma globulariifolium*, the populations from the Pyrenees were found to be genetically indistinguishable from Western Alpine plants, whereas a deep split was found in the Alps separating an Eastern and a Western group along River Etsch/Adige (Schönswetter et al. 2002). In the highly disjunct *Androsace halleri*, the most internal cpDNA haplotype is found in the Cordillera Cantábrica and in the Eastern Pyrenees, whereas the haplotypes from Massif Central and Alsace are derived by one and two mutational steps, respectively, indicating eastward range expansion (Dixon et al. 2007). A similar pattern was suggested in an amplified fragment length polymorphism (AFLP) study of *Pritzelago alpina* (Kropf et al. 2003), where populations from the Cordillera Cantábrica formed the sister group to the remaining populations. Some lineage diversification was observed in the Pyrenees, whereas one lineage was shared among Northeastern Alps, Tatras, and Southern Carpathians. A further clear example of colonization of the Carpathians from the Alps is provided by *Senecio carniolicus* (Suda et al. 2007), where diploid, tetraploid, and hexaploid cytotypes were found in the Eastern Alps, whereas the Carpathian populations (Tatras, Southern Carpathians) were uniformly hexaploid. In the Alpine-Carpathian *Hypochaeris uniflora*, Mráz et al. (2007) detected three strongly differentiated lineages in the Alps and the Western and the Eastern Carpathians, indicating long-term isolation of these three regions from each other. Most of the Alpine range was apparently colonized post-glacially from an Eastern Alpine refugium. By applying AFLP fingerprinting and chloroplast DNA data to *Campanula alpina* distributed in the Alps and Carpathians (mountains of the Balkan Peninsula were not sampled), Ronikier et al. (2008a) detected a deep split between the Alps and the Carpathians. Further splits separated Southwestern, Southeastern, Northeastern, and Northwestern parts of the Carpathian arch, indicating that the phylogeographical pattern of *C. alpina* has been mainly shaped by vicariance events. Virtually no AFLP differentiation was found among Pyrenean, alpine, and Tatra populations of *Oxytropis campestris* (Schönswetter et al. 2004a). In *Bupleurum stellatum*, mainly distributed in the Western Alps and the Southern part of the Central Alps, a deep split was found within the continuous distribution area roughly halfway between Lago di Garda and Lago di Como (Schönswetter and Tribsch 2005). The isolated population(s) from

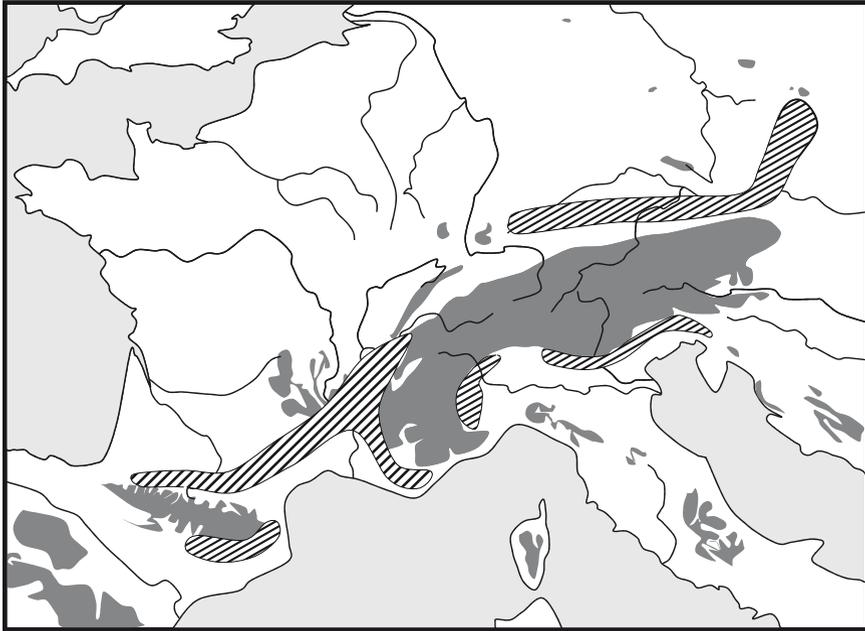
Corsica clustered with high statistical support within the Eastern main group instead of the geographically closer Western group. The presence of several private AFLP fragments in the Corsican population, however, indicated that more time has elapsed since the dispersal event than in cases like *P. globulariifolium* and *O. campestris*. A strongly idiosyncratic genetic structure was revealed by a cpDNA study of *Pulsatilla vernalis* (Ronikier et al. 2008b), distributed over the EMS, parts of the Northerly adjacent lowlands, and Southern Fennoscandia. Two haplotype families separated by several mutational steps were found, both of which occurred throughout the species' distribution area except for the northernmost parts. Interestingly, the centralmost haplotype is essentially found over the entire distribution area, but was lacking from the Alps and Southern Finland. Specific microsatellite variation indicates the occurrence of periglacial lowland refugia North of the Alps, acting as source for post-glacial colonization of Scandinavia. The presence of different fixed haplotypes in Eastern and Western Scandinavia, however, suggests independent post-glacial colonization of these two areas, with possible founder effects.

In comparison with plants, much fewer animal species with disjunct alpine distributions have been analysed so far. A good example, resembling patterns already found in several plant species, was provided by allozyme polymorphisms in the butterfly *Erebia epiphron* (Schmitt et al. 2006). The lineage of *E. epiphron* from the Northern Alps is shared with the Jeseník population in Northern Czech Republic, thus strongly supporting a glacial link between the Northern Alps and the more Eastern Sudety Mountains. However, being currently separated by hundreds of kilometres from the Alps in a geographically small mountainous area, the *E. epiphron* populations in the Jeseník apparently represent a relic of a formerly wider distribution of the species. A link between the Western Alps and the Pyrenees is suggested by a genetic lineage shared by both regions. The Eastern Pyrenees, however, host an endemic lineage of this species underlining the independent evolution of one genetic entity most probably at the Southeastern slopes of the Pyrenees. Interestingly, the same area hosts a strongly differentiated lineage of the plant *A. halleri* (Dixon et al. 2007). Two further genetic groups in *E. epiphron* are endemic to the Southern and Eastern Alps (Fig. 1).

In summary, whereas some phylogeographic studies on species of the EMS provided evidence for highly dynamic scenarios involving unintuitive links among geographically widely separated mountain ranges (such as the link between the Eastern Alps and Corsica in *B. stellatum*, Schönswetter et al. 2005), others suggested vicariance events caused by disruptions during warm stages of the Pleistocene, resulting in splits of varying depths (shallow: e.g., *O. campestris*, Schönswetter et al. 2004a; deep: e.g., *H. uniflora*; Mráz et al. 2007).

## 2.2 Montane Species

Montane species show strong ecologic affinities to the forest belt habitats of the EMS and therefore occur at lower altitudes than the alpine species. However, this biogeographical group is much less studied than the one of alpine disjunct species.



**Fig. 1** Hypothetical distribution patterns of the alpine disjunct butterfly *Erebia epiphron* in Western Europe during the last glaciation. Redrawn from Schmitt et al. (2006)

A good example for a widespread montane species is represented by the butterfly *Erebia euryale*, which is a character species of the mountain forest biome of Southern Europe (Sonderegger 2005). Schmitt and Haubrich (2008) analysed allozyme patterns of 11 populations from four EMS (Pyrenees, Alps, Southern Carpathians, Rila) and found four well-supported groups (i.e., Pyrenees, Western Alps, Eastern Alps and Southeastern Europe, represented by Southern Carpathians and Rila). The genetic diversity of the populations was the highest in the Southeastern European group and decreased step-wise towards the West. Interestingly, the populations from Bulgaria and Romania were almost identical; therefore, the authors assume that they were not separated during the last ice age. In contrast, the differentiation among the three Western Alpine populations was considerable. Consequently, (1) the most important refugial area for this species has been located in Southeastern Europe including at least parts of the Carpathians and the Bulgarian mountains, (2) important refugial areas existed at the Southeastern or Eastern edge of the Alps, (3) scattered populations survived along the Southwestern Alps, and (4) relatively small populations have persisted at the foothills of the Pyrenees (Fig. 2). More studies in species of this distribution type are urgently needed to clarify whether the phylogeographic structure found in *E. euryale*, a character species of the European mountain forest biome, is representative for this biome as a whole or not.



**Fig. 2** Hypothetical distribution pattern of the montane disjunct butterfly *Erebia euryale* in Europe during the last glaciation. Note that these centres only refer to the ones detected in this survey; thus, further centres of survival are possible and reliable, e.g., in peninsular Italy. Redrawn from Schmitt and Haubrich (2008)

### 2.3 *Dinodal Mountain Species*

Species of headwaters of mountain areas are often classified as belonging into an own biogeographic unit, the dinodal (Malicky 1983, 2000). Due to their water dwelling larvae, many Trichoptera species are considered part of this group. The most extensive genetic study of mountain species in this unit was performed on the mitochondrial COI gene of the caddisfly *Drusus discolor* (Pauls et al. 2006), which occurs in the mountain areas of Southern and central Europe.

No close relationships among mountain systems were detected (i.e., strongly differentiated endemic genetic lineages were detected in the Cantabrian Mts., Pyrenees, Massif Central, Eastern Sudety Mts., Tatras, Eastern and Southern Carpathians, as well as the high mountain systems of the Balkan Peninsula). Thus, long-lasting isolation and survival of populations in all these mountain systems over several glacial and interglacial cycles is the most likely scenario, and the strong liaison of the *Drusus* larvae with cold headwater mountain streams might have successfully impeded the exchange of individuals between different high mountain systems throughout time. Genetic differentiation and structuring in *D. discolor* had some similarities with the one observed in the mountain forest species

*E. euryale* (Schmitt and Haubrich 2008), i.e., a Western and an Eastern Alpine lineage, an endemic Pyrenean group, and endemic lineages in Southeastern Europe.

Interestingly, in *D. discolor* all relic populations from the low mountain ranges of central Germany (i.e., Rothaargebirge, Rhön, Harz, Thüringer Wald, Fichtelgebirge, Erzgebirge) share the same genetic lineage endemic to this region. This underlines the inference of an independent centre of survival and differentiation in this region, followed by post-glacial retraction to these mountain areas from lower areas. The same phenomenon was observed for the Jura, Vosges, and Black Forest populations. Finally, the populations from the Bohemian Massif harbour the Eastern Alps lineage, thus giving evidence for a Würm glacial link between these two adjacent regions.

The phylogeographic patterns observed in *D. discolor* show two interesting characteristics: (1) Different high mountain systems do not share closely related lineages, thus rejecting the hypothesis of continuous lowland areas between such widely separated mountains during glaciations; (2) Lower mountain ranges being in some geographic proximity to each other share haplotypes of the same genetic lineage, thus supporting the idea that the lower areas between them allowed high levels of gene flow at least during the last glaciation. Maybe the intermediate lowlands even are the refugia for such genetic lineages.

Another representative of headwaters of mountain areas is the freshwater flatworm *Crenobia alpina*. Allozyme electrophoresis and mtDNA sequencing of this species in the Eastern Alps and Germany unravelled a considerable number of strongly differentiated genetic lineages with their divergence maybe dating back as far as the late Tertiary. Most of these lineages most probably survived the glaciations of the Alps in small peripheral refugia to which some of them might still be restricted. Several of these refugia had their most likely location at the Northern margin of the Alps (Brändle et al. 2007).

### 3 Arctic-Alpine Species

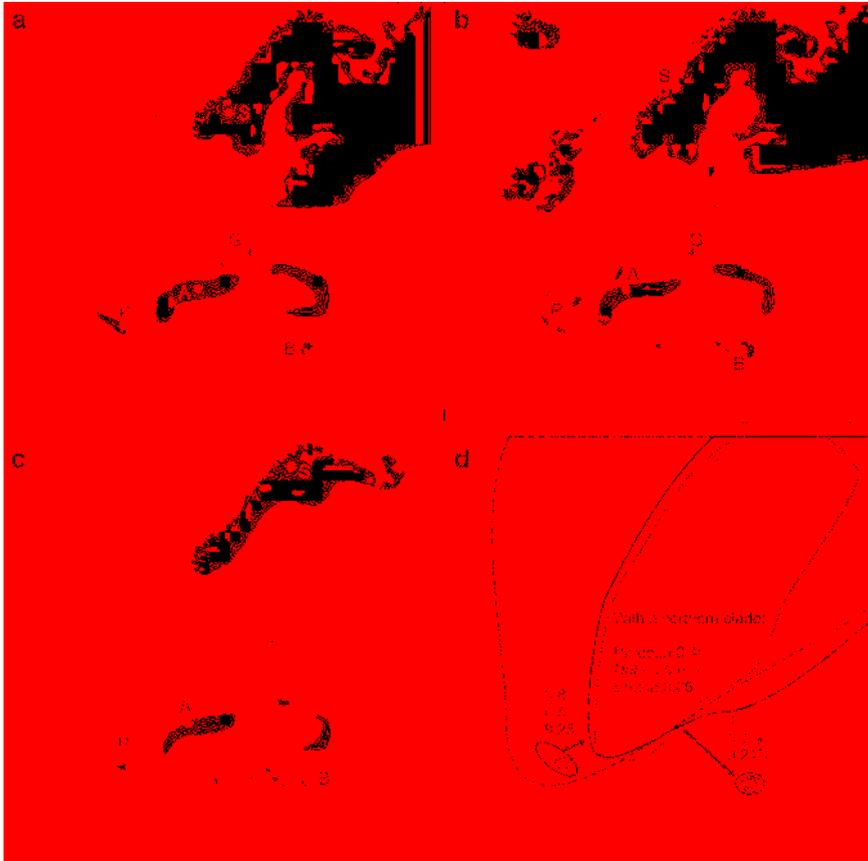
Arctic-alpine distribution ranges comprise the arctic as well as the alpine belt of more Southern mountain ranges. Typically, arctic-alpine distributions are large and often encompass the entire Northern Hemisphere (circumpolar distribution); more restricted ranges are, e.g., amphi-Atlantic or amphi-Pacific (Abbott and Brochmann 2003). The last few years have seen increasing interest in phylogeographic studies of arctic-alpine higher plants, which are by now the doubtlessly best studied group. In the following, we focus on the relationships between the disjunct distribution areas of arctic-alpine taxa in the EMS and their main distribution area in the Arctic.

A close relationship between the central and Northern European populations of a species appears intuitively self-evident and has often been suggested by classical biogeographers (e.g., Vierhapper 1918; Holdhaus 1954; de Lattin 1967; Ozenda 1988). Good examples for such a connection are *Ranunculus glacialis* (Schönswetter et al. 2003), *Arabis alpina* (Ehrich et al. 2007), and *Veronica alpina* (Albach et al. 2006),

which all colonized the North Atlantic region from source populations in the Alps. *Dryas octopetala* likely colonized Northern Europe from source populations located between the Scandinavian ice shield and the Alps (Skrede et al. 2006). In contrast, the Scandinavian populations formed monophyletic groups in *Minuartia biflora* (Schönswetter et al. 2006b) and *Carex atrofusca* (Schönswetter et al. 2006a), indicating that Northern European and Alpine populations have been separated for a certain period of time. In *Carex bigelowii* (Schönswetter et al. 2008), the single Alpine (meta-)population has strong links to the phylogeographical group dominant in the Hercynic mountains, the Tatras, Scandinavia, Iceland, and Scotland. The Hercynic populations were genetically variable, while only a few clones were detected in the Alps, indicating a strong founder effect. Whereas the lack of phylogeographic structure within the four detected phylogeographic groups of *Juncus biglumis* did not allow tracing the closest relatives for Alpine and Scandinavian populations (Schönswetter et al. 2007), *Ranunculus pygmaeus* populations from the two latter areas have their closest relatives on the Taymyr Peninsula in Northern Siberia and in the Urals, respectively, (Schönswetter et al. 2006b). In *Comastoma tenellum* (Schönswetter et al. 2004b), two unrelated lineages were found in the Alps. The by far most common one was endemic to the Alps, while the second, encountered only in two Eastern Alpine populations, exhibited unresolved relationships to Carpathian and Scandinavian populations. Thus, this species has apparently colonized the Alps at least twice.

Similar arctic-alpine distribution patterns like in plants are also observed in many animal species. Holdhaus (1954) listed several dozens of invertebrate species with this distribution pattern in Europe in his seminal monograph on the legacy of the Pleistocene glaciations preserved in contemporary animal distributions. However, while most molecular studies are from plants, mitochondrial sequence variation has been studied in not more than three arthropod taxa of this disjunction type until now.

An illustrative example is provided by wolf spiders of the *Pardosa saltuaria* group (Muster and Berendonk 2006). This is a complex of six closely related species (Wunderlich 1984; Marusik et al. 1996) whose taxonomic status needs revision in the light of the recent genetic findings. The species group is patchily distributed from the Western Palearctic over Siberia to the Altai (Fig. 3a). Focussing on the European range, Muster and Berendonk (2006) found three clades of deep mitochondrial divergence, i.e., a Pyrenean clade, a Balkan clade, and a “Northern clade.” The latter included Scandinavia, the Alps, the Carpathians, the Giant Mountains, and the Bohemian Forest. Recent results from a nuclear rDNA marker (spanning ITS1, 5.8S rDNA, ITS2) generally confirmed this pattern of genetic divergence (Muster et al. unpublished). A detailed population genetic analysis using the information of both loci (mitochondrial and nuclear) in a coalescent-based Bayesian Markov chain Monte Carlo (MCMC) framework (Hey and Nielsen 2007) revealed a pattern of extensive but differential late glacial gene flow among Northern clade areas (Muster et al. 2009). The inferred gene flow was mainly unidirectional with a prevailing direction from the North to the South.



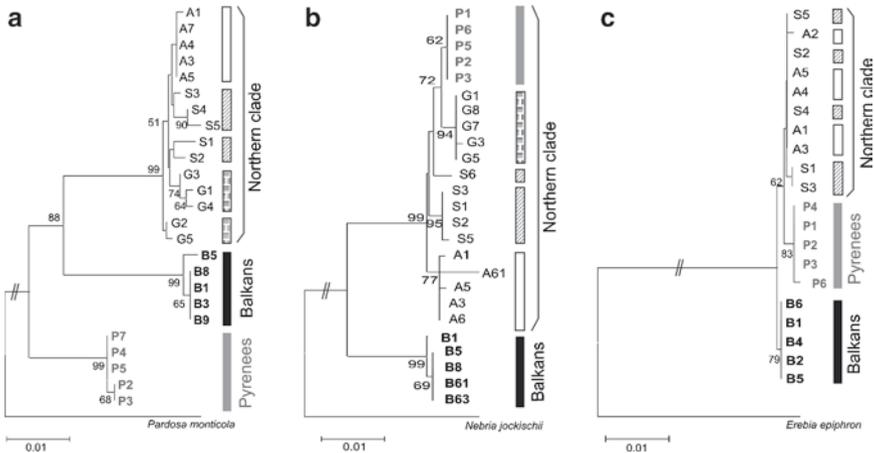
**Fig. 3** European distribution areas and sampling localities of three arctic-alpine invertebrates: the wolf spiders of the *Pardosa saltuaria* group (a) redrawn from Muster and Berendonk (2006), the ground beetle *Nebria rufescens* (b) modified from Holdhaus and Lindroth (1939), and the butterfly *Erebia pandrose* (c) modified from Varga (1971). The white dots indicate the sampling locations. Abbreviations: A Alps; B Balkans; G Giant Mountains; P Pyrenees; S Scandinavia. In Fig. 3d the geographic extension of major mitochondrial clades is shown, together with the Kimura 2 parameter distance (in %) between clades and within the Northern clade. Solid line *Pardosa saltuaria* group, chain line *N. rufescens*, dotted line *Erebia pandrose*

In order to test the phylogeographic hypothesis derived from the wolf spider data, preliminary results from two further arctic-alpine arthropod taxa are presented here. The ground beetle *Nebria rufescens* (Fig. 3b) occupies an even larger arctic area than the *Pardosa* spiders and includes North America (Holdhaus 1954). The area of the butterfly *Erebia pandrose*, on the other hand, is mainly restricted to Europe (Fig. 3c), with a few populations in the mountains of Central Asia (Cupedo 2007). Within Europe, the distributions of these species are also different (Fig. 3a–c), with *N. rufescens* resembling more the boreomontane distribution type of de Lattin

(1967), as indicated by occurrences on the British Isles and in some rather low mountain ranges. In marked contrast, *E. pandrose* shows the typical arctic-alpine disjunction sensu de Lattin (1967), and even in Scandinavia the species' range is limited to the mountains and the far North, while it is restricted to the highest elevations in the Southern European mountains.

In a pilot study, Muster et al. (2009) sequenced the complete mitochondrial ND1 gene from five individuals of each of four (in *E. pandrose*) and five populations (in *N. rufescens*) as shown in Fig. 3. To allow the direct comparison of the level of genetic variation, the data set of the wolf spiders was reduced to the first five sequences of each of five similar areas. Both striking similarities and idiosyncratic differences in the resulting genetic structures were found. First, the overall level of sequence divergence was highly different (K2P-distance: *Pardosa* 2.4%, *Nebria* 1.3%, *Erebia* 0.2%) despite the fact that identical genes in co-distributed species were investigated. Analysis of molecular variance (Excoffier et al. 1992, 2005) clearly showed that the populations in the Southern mountains contributed disproportionately to the overall genetic differentiation of these taxa.

Despite the different life histories and migration abilities in these three taxa, great similarities in the genetic structures existed (Fig. 4). Most importantly, the haplotypes from the Balkans formed a distinct and highly supported clade in all three study systems. Another common feature was the low divergence among Scandinavia, the Alps, and several rather low Central European mountain ranges (Hercynian Mountains), which always clustered in a “Northern clade” with little geographic structure (except for *N. rufescens*, where each area has its own unique mutations).



**Fig. 4** Neighbour-joining trees showing the phylogenetic relationships of 25 individuals of the *Pardosa saltuaria* group (a), 25 individuals of *Nebria rufescens* (b), and 20 individuals of *Erebia pandrose* (c). All three trees are shown on the same scale, highlighting the different degree of overall sequence divergence. Bootstrap values >50% from 1,000 replicates are shown at the nodes. Letter abbreviations are the same as in Fig. 3

The position of the Pyrenees differed among the investigated taxa. While the Pyrenean haplotypes formed a clade as distinct from the Northern populations as the Balkan clade in *Pardosa* and *Erebia*, the Pyrenees clearly fell within the Northern clade in *Nebria*.

It is most likely that the populations from Scandinavia, the Alps, and the Hercynian Mountains are descendants of large populations that have dwelled in the periglacial tundra belt during the last glaciations, or at least that substantial levels of late glacial gene flow among mountain ranges occurred. Similarly, allozyme data from the diving beetle *Hydroporus glabriusculus* suggest a common late glacial origin of the British and the Scandinavian populations from source populations South of the Northern European ice sheet (Bilton 1994). In the *Nebria* beetles and in the arctic-alpine burnet moth *Zygaena exulans* (Schmitt and Hewitt 2004), such late glacial connections obviously existed also between the Alps and the Pyrenees. Thus, the traditional concept of enormous continuous distributions in the periglacial tundra belt during the glaciations (Holdhaus 1954; de Lattin 1967) could be partly corroborated. On the other hand, the Balkans and in some taxa also the Pyrenees have been isolated, and populations there consequently evolved independently for fairly long periods of time, probably since several glacial cycles. They thus represent old relic populations of high conservation importance. Altogether, the populations from disjunct parts of the distribution areas of arctic-alpine disjunct arthropods can be regarded as relics, which originated at very different time horizons.

#### 4 Relics or not Relics: That's the Question

The genetic analyses of different species with alpine and arctic-alpine disjunct distribution patterns unravel a great variety of different biogeographical patterns and considerable differences in the time frames of the vicariance and/or dispersal events. Most often, separations in arctic-alpine species between the Northern populations and the high mountain systems in central and Southern Europe (often the Alps) are recent (i.e., post-glacial) phenomena. In alpine disjunct and in arctic-alpine species, adjoining high mountain systems often own the same genetic lineage (as, e.g., shown by multiple genetic links between the Western Alps and the Pyrenees, the Eastern Alps and the Carpathians, and the Southeastern Alps and the Dinaric mountain systems), thus underlining a recent (in most cases post-glacial) vicariance or dispersal event in the respective genetic lineage. A similar situation is often found in high and low mountain systems in geographic proximity (e.g., Northeastern Alps and Bayerischer Wald) or in neighbouring low mountains, e.g., in Germany. These areas thus harbour young relic populations that may result from large zonal distributions of such taxa in the tundra belt during the last ice age.

The most basal and thus oldest splits in arctic-alpine and alpine disjunct species are commonly found in the Southernmost European high mountain systems with typical alpine zonation. The Cordillera Cantábrica in this respect represents one important

mountain area for the survival of old genetic entities, but also the high mountain systems of the Balkan Peninsula and to some degree the Pyrenees show importance as areas for the survival of old lineages, which can be interpreted as relics of cold stages of a more distant past.

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# Linking Genetics and Ecology: Reconstructing the History of Relict Populations of an Endangered Semi-Aquatic Beetle

Andrea Matern, Claudia Drees, Alfried P. Vogler, and Thorsten Assmann

**Abstract** Population ecology and genetic studies are complementary approaches to address central questions of conservation biology and can provide information for the protection of biodiversity and the improvement of conservation measures which may otherwise be unattainable. This contribution highlights the benefit of combining diverse approaches for obtaining knowledge on a relict species and for implementing suitable conservation measures. We use the example of *Carabus variolosus*, a flightless European beetle that is listed as a Species of Community Interest in the EU Habitats and Species Directive. Ongoing investigations established the species' habitat choice, population ecology and population genetics, and yield first results from mitochondrial DNA analysis (COI). In small isolated habitat patches we found small sized populations that are genetically strongly differentiated even if adjacent. Evidence for a number of glacial refugia was found. However, all *C. v. nodulosus* populations studied North of the Alps share the same haplotype. We will discuss our findings in the light of the history of the species, of its taxonomic classification and of their implications for conservation.

## 1 Introduction

The flightless ground beetle *Carabus variolosus* Fabricius, 1787 (Coleoptera: Carabidae) is a rare and threatened habitat specialist of headwater areas and swamps in deciduous woodlands. Both larvae and adults live close to the water edge where

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they forage (Sturani 1963). Populations persist in localised areas under pristine habitat conditions of which *C. variolosus* is considered to be an indicator (Turin et al. 2003). Remaining populations are spread in a disjunct range throughout Central and South Eastern Europe. While already confined to a restricted range, the distribution of *C. variolosus* is further contracting presumably due to habitat destruction and pollution (Pavicevic and Mesaros 1997; Turin et al. 2003; Matern and Assmann 2004), resulting in its status as a “relict species” (sensu Fryxell 1962).

Taxonomically, *C. variolosus* has been subdivided into two allopatric subspecies, which can be distinguished reliably only by the different tip shape of the male genitalia (Breuning 1926). The Western *C. v. nodulosus* Creutzer, 1799 extends to Germany and France and along the Dinaric mountains Southwards to Macedonia (Breuning 1926; Turin et al. 2003). The Eastern *C. variolosus* s. str. ranges from the Czech Republic and South Poland along the Carpathian mountains to the Western edge of the Balkan mountains in Bulgaria (Breuning 1926; Turin et al. 2003). Over the past 20 years some authors considered both taxa as sister species (e.g. Casale et al. 1982; Deuve 1994), triggering a debate about their taxonomic rank. In 2004, *C. variolosus* was listed as a “species of Community interest” under the EU Habitat and Species Directive, which requires the European member states, to secure its long-term survival. This may be achieved by strict measures for protection, including the designation of reserves, the application of suitable management plans, and the establishment and maintenance of a “favorable conservation status” for the species (The Council of the European Communities 2004). However, expanding basic knowledge about this rarely studied beetle is necessary to assess and monitor its conservation status and devise effective management measures.

Population ecology and population genetics are complementary approaches to address questions about species conservation management. Combined, they offer valuable insights into viable population size and structure, population dynamics and isolation, dispersal and colonization ability, while addressing different spatial and temporal scales (e.g. Ranius 2006; Schmeller and Merilä 2007; Schwartz et al. 2007). This is of great importance for the protection of biodiversity and the improvement of conservation measures, providing information on, for example, the habitat requirements of species (e.g. Gröning et al. 2007), the levels of genetic variability in populations (e.g. Böhme et al. 2007), and the recognition of distinct gene pools and evolutionary lineages (e.g. Palsbøll et al. 2007).

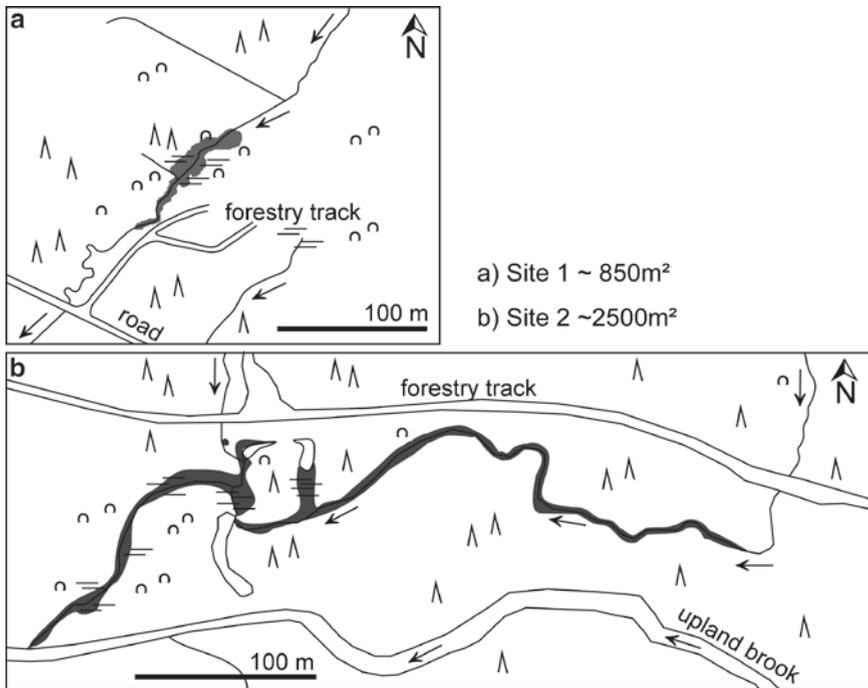
Here, we review our ongoing investigations on the population ecology and population genetics of *C. variolosus*, including recent results from mtDNA data. We discuss the findings with a specific focus on the (1) postglacial history of the Western subspecies *C. v. nodulosus* and (2) the implications of the results for conservation issues showing the benefit of combining field-based and genetic approaches.

## 2 Habitat Choice and Demography

Demography and habitat choice of *C. variolosus* were investigated in a 2 year mark-recapture study at two representative relict populations of *C. v. nodulosus* in North-Western Germany. Parameters determining the habitat preferences of adult beetles

were estimated from habitat suitability models (e.g. Matern et al. 2007). These studies have highlighted and further refined which specific requirements determine habitat associations (Matern et al. 2007). Key habitat variables governing the occurrence of the adult beetles were found to be: short distance to water, high soil moisture, open woodland vegetation cover, a near-neutral pH of the soil and the lack of ground cover. Accordingly, the extent of suitable habitat was rather restricted (Fig. 1). Continuous observations of individual beetles confirmed their confinement to regions of wet soil and close to, or covered by, water (Drees et al. 2008).

Mark-recapture data showed *C. variolosus* to be a spring breeder in the sense of Larsson (1939), i.e. reproduction took place after the emergence of overwintering adults between April and June. Estimates of population sizes at both sites were very low (Matern et al. 2008). During the spring activity period in 2004 the total number of different adults captured amounted to 63 (Site 1) and 161 (Site 2) individuals, corresponding to a maximum of 150 (Site 1) and 215 (Site 2) individuals when corrected using the Jolly–Seber estimate (Jolly 1965; Seber 1965). Population densities, however, estimated at 1.75 (Site 1) and 0.85 (Site 2) individuals per 10m<sup>2</sup>, were normal to fairly high in comparison to more common species of *Carabus*



**Fig. 1** Habitat preference of two *C. variolosus nodulosus* populations in the Forest of Arnsberg, Northwestern Germany. Occupied habitat is represented in grey. Figure 2 shows the location of these sites. (a) Population 1, (b) Population 2

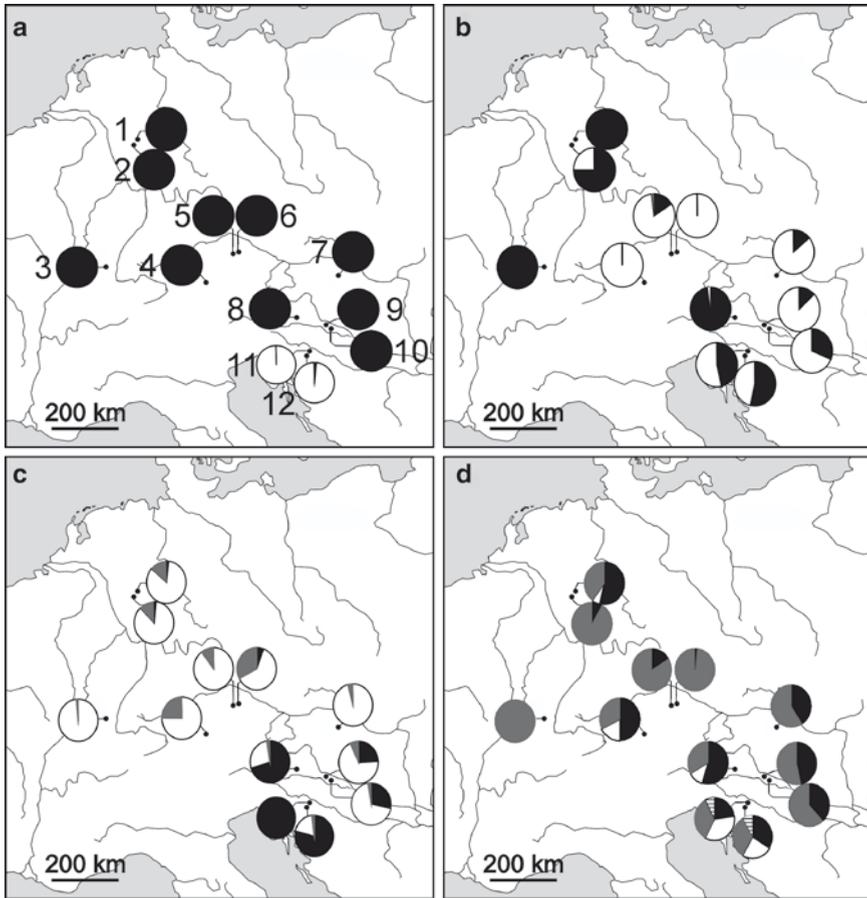
(Matern et al. 2008). Hence, the total population sizes appear to be limited by the extent of suitable habitat, rather than low density. This illustrates the imminent threat to population survival from mechanisms operating in small habitat patches (extinction vortex, Gilpin and Soulé 1986). However, adults were found to be long-lived and almost 30% of the reproductively active individuals were at least in their second breeding period (Matern et al. 2008). This reduces risks of reproductive failure from climatic variation or other stochastic factors in any given year.

### 3 Genetic Population Structure According to Nuclear Genes

Genetic analysis using cellulose acetate electrophoresis was performed on 12 populations of *C. v. nodulosus* throughout most of its distributional range, and including those used for mark-recapture studies above. A total of 308 specimens were included in the analysis for a mean sample size of 25 individuals and 16 allozyme loci (Matern et al. 2009; Fig. 2). Despite small population sizes (inferred from small areas of suitable habitats at most sampling sites), we found no evidence for inbreeding, as populations did not deviate significantly from Hardy–Weinberg equilibrium (GENEPOP 3.4, Raymond and Rousset 1995). However, allele numbers and the amount of polymorphism per population were low, with only eight of the investigated loci found to be polymorphic and a maximum of seven polymorphic loci per population, indicating genetic impoverishment.

Population differentiation and correlation between genetic and geographical distances between samples were quantified with the Arlequin package (Excoffier et al. 2005). Despite the low number of private alleles, populations were highly differentiated (overall  $F_{ST} > 0.45$ ) owing to locally differentiated allele frequencies among populations, as exemplified by Fig. 2. This finding supports the possibility that populations were affected by genetic drift due to small population sizes. Neither clinal variation nor a directional loss of alleles that could hint at any postglacial recolonization scenario according to the allele elimination hypothesis (Reinig 1938; Hewitt 1999) could be detected. Generally, geographic distance only had a marginal influence (<10%) on the isolation of populations (Mantel test). A neighbor-joining tree based on genetic distances among all *C. v. nodulosus* populations confirms this result, showing poor agreement between genetic and geographical distance across the entire range (not shown). Even neighboring populations at distances of only 2–3 km in the same forest and drainage system were found to be virtually independent of each other as shown, for example, by significant pairwise  $F_{ST}$  values of 0.27 and 0.14 between populations 1 and 2 and populations 5 and 6, respectively.

Strongly separated were two populations from Southern Slovenia (sites 11 and 12) which showed deviating allele frequencies and some private alleles compared to the other investigated populations. This is confirmed by Bayesian structure analysis (STRUCTURE, Pritchard et al. 2000) which generally assigns the individuals of these two populations to one distinct genetic cluster if no a priori assumptions of geographical origin are made (Matern et al. 2009). This pattern



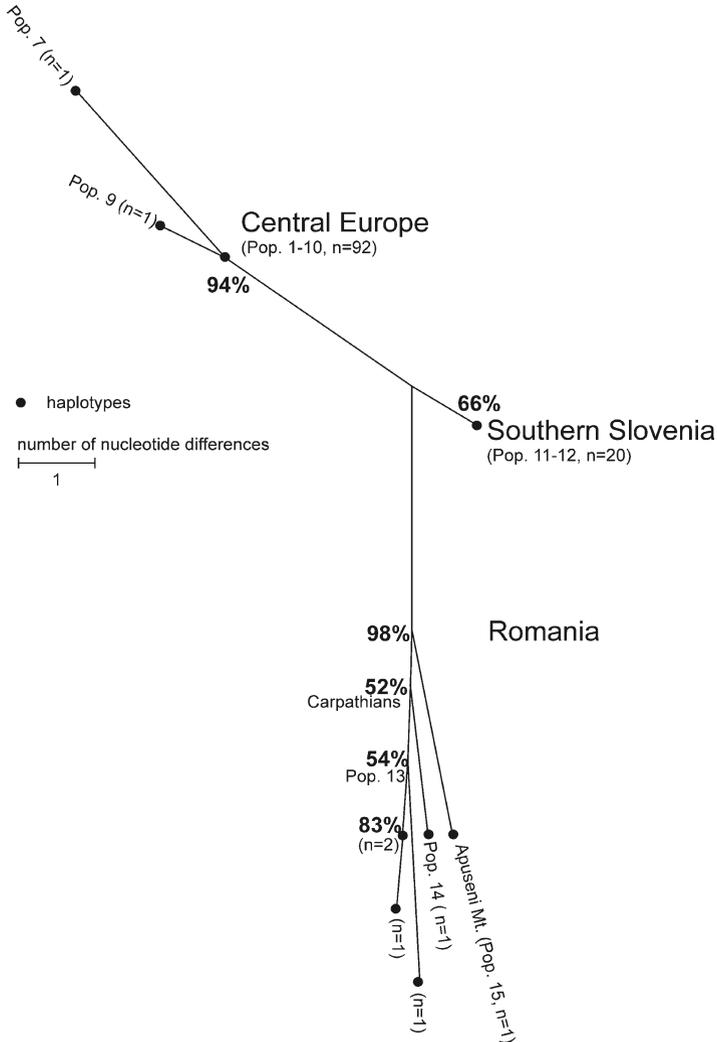
**Fig. 2** Allele frequencies of (a) alcohol dehydrogenase, (b) glucose-6-phosphate isomerase, (c) aspartate aminotransferase, and (d) mannose-6-phosphate isomerase in the investigated populations of *C. variolosus nodulosus*. Different colours indicate various alleles at the respective locus

may be caused by genetic drift and an efficient present isolation between the Southern Slovenian samples and the remaining Central European populations that might have existed since the survival of these populations in different glacial refugia (cf. Chaps. 4 and 5.1, this contribution).

#### 4 Mitochondrial DNA Data

Mitochondrial DNA sequences largely confirmed the results from allozyme studies, also indicating minimal within-population variation and the separation of the South Slovenian samples. We amplified a fragment of the cytochrome oxidase I (COI)

gene using primers C1-J-2183 (Jerry) and TL2-N-3014 (Pat) (Simon et al. 1994) and successfully sequenced 608 bp for 114 specimens of *C. v. nodulosus* and six individuals from three Romanian populations of *C. v. variolosus*. Only nine different haplotypes were identified (Fig. 3). There was hardly any haplotype variation within populations of *C. v. nodulosus*. With the exception of two individuals deviating by one and three nucleotide changes, respectively, all remaining 92 individuals



**Fig. 3** Neighbor-joining tree (Saitou and Nei 1987) based on the number of nucleotide changes between 120 sequences of COI fragment. Numbers at nodes refer to support from 1,000 bootstrap replicates. Central European and Southern Slovenian specimens: *C. v. nodulosus*, Romanian specimens: *C. v. variolosus*. Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007)

in the Central European populations (1–10) shared the same haplotype. The 20 individuals from Southern Slovenian (populations 11 and 12) shared a single haplotype that differed from the former by four nucleotide changes. All of these were separated from *C. v. variolosus* haplotypes by at least four nucleotides (Fig. 3). This finding corroborates that the Southern Slovenian populations of *C. v. nodulosus* survived in a different glacial refugium than the remaining European samples (cf. Chap. 5.1, this contribution).

## 5 Synthesis

### 5.1 *C. variolosus*: A Relict Species

In contrast to other weak dispersers that survived the ice ages in Southern refugia and remained largely confined to these areas ever since, *C. variolosus* expanded its range subsequent to postglacial climate changes despite being flightless. As a stenotopic woodland species specialised on water margins, *C. variolosus* must have suffered considerably from the climate changes of the glacial periods. Given the species' current habitat preference (e.g. Sturani 1962), its confinement to lowland or lower montane altitudes (e.g. Breuning 1926; Barloy et al. 2004) and its strict stenotopy established by the habitat models (Matern et al. 2007), it is likely that populations survived the ice ages in sheltered, wet deciduous woodlands. These habitats may have persisted during the Last Glacial Maximum in Iberia, Italy and the Balkans (Zagwijn 1992), and in the Carpathians (Kotlík et al. 2006). Deffontaine et al (2005) furthermore suggest the existence of mixed coniferous-deciduous forests in the river systems present near the Alps and in the Hungarian plain and Slovakia. Paleobotanical and genetic evidence also corroborates the survival of beech populations as far North as Southern France, the Eastern Alps, Slovenia, Istria and possibly Southern Moravia-Bohemia (Magri et al. 2006), but so far no refuges of forest-forming tree species are known from further North. Recent faunistic records of *C. variolosus*, however, exist from numerous regions North of the Alps such as the presence of *C. v. variolosus* at the foothills of the Sudetic Mountains, Upper Silesia and the Lublin Upland in Southern Poland (Pawlaczyk et al. 2004) and as far as the Hamburg area, where *C. v. nodulosus* occurred until about 100 years ago (Breuning 1926).

The homogeneous mtDNA in the Central European populations of *C. v. nodulosus* is consistent with a recent colonization of the region North of the Alps. Under the standard molecular clock for insect mtDNA of approximately 2.3% divergence per million years (Brower 1994), all Central European populations of *C. v. nodulosus* and the Southern Slovenian lineage separated no more than 0.3 Myrs ago (0.7% pairwise average sequence divergence). Likewise, the Central European populations and haplotypes of the subspecies *C. v. variolosus* separated approximately 0.5 Myrs ago (1.2% divergences). The existence of these lineages which likely predate

the most recent glacial cycles may suggest the existence of more than one glacial refuge or source area within or near to the investigated area.

Post glacial colonization processes are not reflected in allozyme variability. We did not observe a directional loss of alleles according to the allele elimination hypothesis (Reinig 1938; Hewitt 1999) nor any regionally specific sets of allozymes and their combination in intermediate populations, which could suggest potential contact zones. The largely random geographic structure of allozyme variants, the lack of private alleles, and the lack of correlation in allele frequencies with geographic distance (Matern et al. 2009) suggests that the variation in Central European populations was more similar at first, but that populations lost variability subsequently due to stochastic processes. Hence, the case of *C. variolosus* could represent an example where postglacial colonization, in combination with subsequent geographic isolation, produced genetic differentiation of a similar magnitude as that resulting from survival in different glacial refuges. For example, the postglacial *C. variolosus* populations from Germany and the Vosges display an  $F_{ST}$  of 0.45 (7 polymorphic enzyme loci). This is similar to values obtained for 29 populations of *Carabus punctatoauratus* in the Pyrenees that survived in different glacial refugia (mean  $F_{ST}$  of 0.23, enzyme loci, Assmann and Weber 1997) or to values found in *C. solieri*, another flightless silvicolous ground beetle, that remains largely confined to its glacial refuges in the Southwestern Alps close to the border between France and Italy ( $F_{ST}$  of 0.34, microsatellite markers, Garnier et al. 2004). Although  $F_{ST}$  values obtained with different makers are not directly comparable, the latter example also supports the depth of population separation within the Northwestern lineage of *C. variolosus*.

It is likely that the present interpopulation differentiation of *C. v. nodulosus* is only partly due to low dispersal power of these flightless beetles, but was exacerbated by small population sizes. Ecological knowledge helps to understand better what affects the recent population history of *C. v. nodulosus*: its high habitat specificity not only increases the barrier function of the landscape structure surrounding suitable habitat patches but may also result in very small populations, as demonstrated for the above two populations in Northwestern Germany (Matern et al. 2008). Descriptions of patch sizes in additional populations of *C. v. nodulosus* suggest that these may exist or have existed under similar spatial conditions (e.g. Gersdorf and Kuntze 1957; Perraudin 1960; Morati and Huet 1995). While the investigated populations do not appear to be inbred, they are genetically impoverished in comparison with other carabid beetles (Matern et al. 2009). This renders them highly susceptible to genetic stochasticity that is associated with extinction vortices, in particular in the face of expected climatic changes.

While *C. v. nodulosus* was distributed much more widely until about a century ago, it has since suffered from a marked decline and nowadays only persists in very localised relict populations (Breuning 1926; Turin et al. 2003; Matern and Assmann 2004). Intensive human land use with its massive impact on the natural hydrological budget is thought to be responsible for the decline (e.g. Perraudin 1960; Weber and Weber 1966; Pavicevic and Mesaros 1997; Turin et al. 2003). The resulting destruction and degeneration of suitable habitat has aggravated the geographical separation of headwater areas and pristine forest swamps that already affected

*C. variolosus* populations under natural conditions but is now increasing the effect of habitat fragmentation. Currently, *C. v. nodulosus* is considered endangered throughout its entire range (Turin et al. 2003), and its highly restricted distribution is further contracting. Therefore, it is unquestionably a relict in the sense of Fryxell (1962) meeting also further specified criteria such as a reduced variability and narrow ecological tolerance, which potentially indicate a reduction of adaptability, and may ultimately be a candidate for extinction. While the beginning of the decline of *C. v. nodulosus* cannot be clearly identified, the relict status of *C. v. nodulosus* in Central Europe, however, is relatively recent, as the beetle successfully colonized this area post glacially. While the situation of *C. v. variolosus* apparently is less alarming (Turin et al. 2003; Pawlaczyk et al. 2004), this subspecies is also characterized by a narrow ecological tolerance and a restricted range that is further declining in some regions (Turin et al. 2003).

## 5.2 Implications for Conservation and Future Work

The example of a species that survived past climate change in refuges and benefited from reverse climatic changes by range expansion despite low dispersal power may give hope for conservation. However, *C. v. nodulosus*, like many other postglacial populations, shows reduced genetic variability (Hewitt 2001), which implies certain risks for long-term survival. Hence, the maintenance of genetic diversity within species is one important goal of biodiversity conservation.

As the Southern Slovenian populations are a distinct genetic unit holding genetic diversity not found in Central European populations, they should consequently be granted special protection alongside the Central European samples. To conserve the remaining genetic diversity, as many of the relict populations as possible should be targeted, since more than 45% of the total allozyme variance of *C. v. nodulosus* is found among populations (Matern et al. 2009). Where possible, a cautious enlargement of the habitat patches may also be attempted.

If further populations within or close to the core of the subspecies range could be found that display high genetic diversity (owing to their location in glacial refuges, for example), these of course would have an especially high conservation priority. Locations of all current relict populations of *C. variolosus* (and of other flightless, sensitive species) may also be an indicator of unique environmental conditions not affected by disturbance and demonstrate the general continuity (stability) of habitats. These occurrences can therefore be used to identify valuable habitats for the conservation of entire species communities.

This study provides an example of increased knowledge and sensible conservation strategies that were derived from a combination of diverse methodological approaches offering different perspectives. The contribution of ecological knowledge is essential where knowledge on a species is scant. Details on the habitat preference and habitat use of *C. variolosus* are crucial for an assessment of factors influencing the incidence and distribution pattern of the species. In the case of

*C. variolosus* this information especially emphasises the urgency to assess population sizes and the necessity to enlarge populations. The analysis of COI provides valuable information on long-term population history that was not available from allozyme analysis. Moreover, it strongly supports the allozyme results which already suggested that the Southern Slovenian populations are a differentiated entity, with relevance for conservation (Moritz 1994). The identification of differentiated population groups is considered crucial for the conservation of the genetic diversity of species (e.g. Ryder 1986; Moritz 1995) and provides important data in rescue efforts of species by means of translocations (e.g. Avise 1996). While mtDNA sequence data contribute additional information on population differentiation and genetic diversity, they should not be used exclusively in the discussion of conservation priorities. It is the population genetic approach with nuclear markers that offers an advanced insight into the isolation of populations and the level and overall distribution of genetic diversity within and across populations. The allozyme results underline the need for recognition of distinct Management Units even at adjacent sites within the same drainage system.

In the context of the EU Habitats Directive and Natura 2000 Network it is now important to extend analyses to the Eastern subspecies *C. v. variolosus*. Further allozyme and DNA analyses to unravel the phylogeny and discover subspecific population structure will show whether the conservation measures established for *C. v. nodulosus* are also valid for *C. v. variolosus*. One important clue, however, is already given by the current COI analysis: sequence divergence between the two taxa is surprisingly low. As the amount of sequence divergence is extremely variable in different lineages, it is impossible to apply a general threshold value to justify taxonomic distinction at the species level (Vogler et al. 1993). Some molecular taxonomists have (controversially) used a 3 or 2% COI divergence for species delimitation across a wide range of organisms including insects (cf. Duckett and Kjer 2003; Hebert et al. 2003, 2004; Moritz and Cicero 2004). Comparisons of mtDNA studies find sequence divergence of less than 2% primarily on an intraspecific level (Vogler and DeSalle 1994; Avise 2000; Woodcock et al. 2007). This is also the case among populations of another ground beetle species, *Nebria rufescens*, which are not formally distinguished at the species or subspecies level and display a mean mtDNA-sequence divergence (ND1) of 1.3% (Schmitt et al. this book). Although the two taxa of *C. variolosus* are “diagnosable” under a phylogenetic species concept (Vogler and DeSalle 1994), their divergence is low and only slightly greater than the striking separation of the Central European and Southern Slovenian populations. This finding may support the older classification as subspecies (cf. Breuning 1926), settle the past discussion on the potential species rank of *C. v. nodulosus* and counteract taxonomic inflation (cf. Isaac et al. 2004).

Future genetic assessment of *C. variolosus* will be necessary to study the success of conservation recommendations such as population increase or population connectivity and thus augment ecological monitoring. Moreover, it may also enhance our knowledge on the relationship between population sizes and the maintenance of genetic diversity and probably their influence on the persistence of these populations.

**Acknowledgements** We are grateful to Božidar Drogenik, Daniela Eisenacher, Jens Günther, Michael Franzen, Istvan Mathé, Wolfgang Paill, Laszlo Rakosy, Annette Ehrhardt-Renken, and Marco Zimmermann for their assistance in sampling and valuable exchange of information and to Dietrich Mossakowski for providing us with an additional specimen from Romania. For technical assistance in the lab we thank Marianne Peters, Beatrice Dames, and Gunnar Siedenschur. We cordially thank Anna Papadopoulou at the Natural History Museum London, who helped with mtDNA sequencing and analysis. We also thank an anonymous reviewer for valuable comments on the manuscript. A. Matern was funded by the German Federal Environmental Foundation (DBU).

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# Peripheral Relict Populations of Widespread Species; Evolutionary Hotspots or Just More of the Same?

Anna Cassel-Lundhagen

**Abstract** Relicts are species with a former, more widespread distribution range but can also include specific populations in parts of a species distribution (thus called relict populations). Such populations are often found towards the species range limits where conditions may be less optimal than in the more central parts. For peripheral relict populations to be valuable for conservation from a biodiversity point of view, they should be exposed to selection pressures that are different from the more central ones allowing for unique adaptations to arise. I will discuss data from studies of two closely related butterfly species where the aim was to evaluate the uniqueness of marginal relict populations in relation to their more central counterparts. Neutral genetic variation and values of adaptive wing traits among populations of the scarce heath (*Coenonympha hero*) and the pearly heath (*C. arcania*) from three biogeographically different regions were compared representing one central and two peripheral parts of their distributions; one peripheral connected to and one isolated from the main area of their distributions. Individuals from the isolated region were significantly differentiated from the peripheral and central regions in both neutral genetic traits and adaptive wing traits, while the peripheral and central populations showed no signs of differentiation in both trait types. I will discuss the possible mechanisms behind the observed patterns and show that the peripheral, relict populations of also widespread species can contribute with valuable and unique genetic variation. Marginal relict populations constitute ideal study objects of evolution in marginal habitats as they can be used to test how well theory and laboratory experiments reflects more complex natural systems.

## 1 Introduction

Peripheral populations at the margin of a species' range may be exposed to ecological and evolutionary processes that differ from more central parts of its distribution. If the level of genetic differentiation is high due to exposure to diversifying

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processes, like random genetic drift and selection, this could eventually lead to speciation (Garcia-Ramos and Kirkpatrick 1997; Mayr 1954, 1963). Thus, such peripheral populations may be unique and important from a conservation point of view (Channell and Lomolino 2000; Lesica and Allendorf 1995).

Relicts are defined as species with a former more widespread distribution range but can also include specific populations in parts of a species distribution (thus called relict populations). Such populations are often found towards the species range limits where conditions may be less optimal than in more central parts (i.e. Gullberg et al. 1998). One could argue that peripheral relict populations of globally widespread species should not be prioritized for conservation as they constitute only a marginal fraction of the species in the global biodiversity. However, both evolutionary history and local selection pressures may differ along a species distribution range and this can lead to unique genotypes and adaptations that are not present elsewhere (Eckert et al. 2008). Especially, peripheral populations of species with limited dispersal capacity are likely to originate from populations that have gone through recurrent bottlenecks during colonization and differentiation can then be rapid and significant if founders are few (Chakraborty and Nei 1977). If the newly established populations lack subsequent gene flow from their sources such effects are expected to remain and accentuate in the region. In addition to the influence of history, genetic drift is likely to be more pronounced towards the range periphery due to a greater influence of stochastic processes. Reduced habitat density as well as climatic constraints will cause size fluctuations of local populations that could cause significant influence of genetic drift with changed allele frequencies as a consequence.

Random effects due to founder events and population fluctuations will lead to changes in the neutral or nearly neutral traits. However, for peripheral relict populations to be valuable for conservation, they should also be exposed to selection pressures that are different from the ones of more central positions (Lesica and Allendorf 1995). If conditions are equal in all parts of a species range or selection pressure is identical, then these populations could differ significantly in neutral markers, while being identical in selected characters. If conditions instead are diverging, selection will favor genotypes that are different from the best adapted ones in the more central regions (Helgadóttir and Snaydon 1986) causing increased global biodiversity.

Diverging selection pressures may be due to a number of factors and can include differences in local or regional climate, species compositions or landscape structures. In the case of landscape structure, for example, selection pressures can act in favor of increased dispersal capacity (Clare and Hughes 2007) as well as against it (Dempster 1991). With increased isolation among patches and a matrix of low quality (lacking for example resting and foraging sites) dispersers may experience increased mortality leading to selection acting against dispersive genotypes (Cody and Overton 1996; den Boer 1990; Heino and Hanski 2001; Schtickzelle et al. 2006). Such altered behavior is likely reflected also in the individuals' phenotype (Berwaerts et al. 2006, 2002; Davis 1984; Dempster 1991; Fric and Konvička 2002; Merckx and Van Dyck 2006; Thomas et al. 1998). For example, short and broad

wings are expected to favor slow, agile flight and appear to be more common among forms that fly in restricted spaces and dense vegetation (Betts and Wootton 1988).

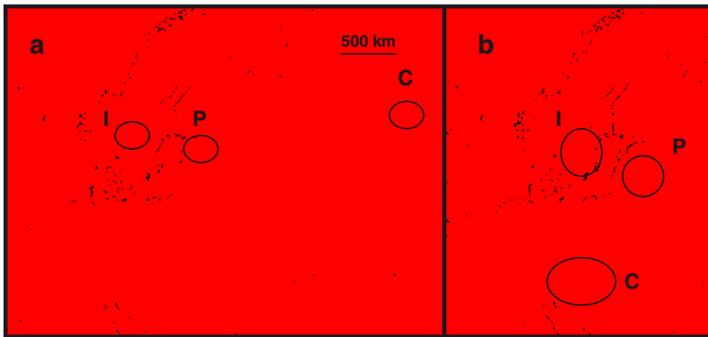
However, if gene flow from central parts to the periphery is substantial, diversifying selection pressures may be hidden due to swamping (Garcia-Ramos and Kirkpatrick 1997). This can occur even if the individual dispersal capacity is equal along the whole range due to a larger net inflow of immigrants into a region with smaller population sizes. Such confounding factors will make discrimination between homogenizing gene flow and lack of diversifying selection problematic and a study setup is needed to allow discrimination among these forces. Studies on peripheral populations should, therefore, be performed including those connected to the main area of the species distribution in some regions and isolated from more central parts in others.

## 2 Examples From Two Heath Butterfly Species

I will discuss data from studies of two closely related butterfly species where the aim was to evaluate the uniqueness of marginal relict populations in relation to more central counterparts. Patterns in neutral genetic markers and values of adaptive wing traits among populations of the scarce heath species (*Coenonympha hero*, Linnaeus, 1761) and the pearly heath species (*C. arcania*, Linnaeus, 1761) from three biogeographically different regions were compared; one central (central Russia for *C. hero* and central Europe for *C. arcania*), one peripheral but connected to the main area of distribution (the Baltic states for both species and termed *peripheral region* in the following text) and one peripheral and isolated (Sweden for both species and termed *isolated region* in the following text) (Fig. 1a,b).

The Scandinavian populations of *C. hero* are relicts of a former more widespread distribution within the Scandinavian peninsula (Berglind 1990, 2007) and the species is therefore classified as “near threatened” in the Swedish Red Data Book (Gärdenfors 2005). On a global scale *C. hero* is widespread, although locally threatened, with a distribution ranging from Japan in the East through central Russia to central Europe in the West. The distribution is fragmentary in Western and Northern Europe and the species is considered threatened in most countries (Eliasson 2005; Kudrna 2002; Tolman and Lewington 1998).

*C. arcania* has a similar distribution as *C. hero* with peripheral populations in the Baltic states and Scandinavia, with the difference that it is widely distributed in Europe, from the Northern Mediterranean to South-central Scandinavia and central Estonia (Kesküla 1992; Kudrna 2002). In the East, it is found in Turkey, in Southern Russia and central parts of the Ural Mts. and Transcaucasia (Kudrna 2002; Tolman and Lewington 1998). Although the Swedish populations of *C. arcania* represent no typical relict due to their distribution being more stable in Scandinavia, they can serve as an illustrating example and a replicate within the described study frame.



**Fig. 1** Distribution range (marked in *light grey*) of (a) the scarce heath butterfly (*Coenonympha hero*) and (b) the pearly heath butterfly (*Coenonympha arcania*) in Europe and western Asia. *Dark grey circles* indicate sampling sites of both species representing central (C), peripheral (P) and isolated (I) parts of their distributions

In this study design the aim was to evaluate the patterns found not only in the light of the relative geographic locations of the populations, but also on their history inferred from neutral genetic marker. I discuss results obtained from selectively neutral traits, i.e. allozymes (Besold et al. 2008; Cassel and Tammaru 2003), and traits under selection represented by traits describing wing morphology (Cassel-Lundhagen et al. 2009). The allozyme data presented here for *C. arcania* are based on a subset of populations included in Besold et al. (2008) to make the data sets in each region comparable.

### 3 Gene Flow

Interestingly there were no indications of gradually reduced levels of genetic variability and gradually increased levels of differentiation from the centre via the peripheral to the isolated region. Instead, the populations in the isolated region had considerably lower levels of variation, while populations in the peripheral region did not differ from the more central ones (Table 1, Fig. 2a,b). Further, the populations in the peripheral populations were only marginally differentiated from the central ones, while the populations in the isolated region were markedly differentiated from both the peripheral and central ones (Table 2). This strongly suggests that the observed pattern is due to a combination of the peripheral and isolated populations' post-glacial colonization histories and their current position. Gene flow among central and peripheral parts appears to be significant enough to maintain a high level of genetic diversity and low differentiation, while lack of gene flow will cause both reductions in variability as well as differentiation. Thus, having a peripheral position per se does not necessarily lead to genetic loss and differentiation.

**Table 1** Comparison of allozyme diversity measures for *C. hero* and *C. arcania* in a central, a peripheral and an isolated region of the two species distributions. Measures include percentage of polymorphic loci where the frequency of the most common allele does not exceed 0.95 ( $P_{95\%}$  loci), allelic richness, observed heterozygosity ( $H_{obs}$ ) and expected heterozygosity ( $H_{exp}$ )

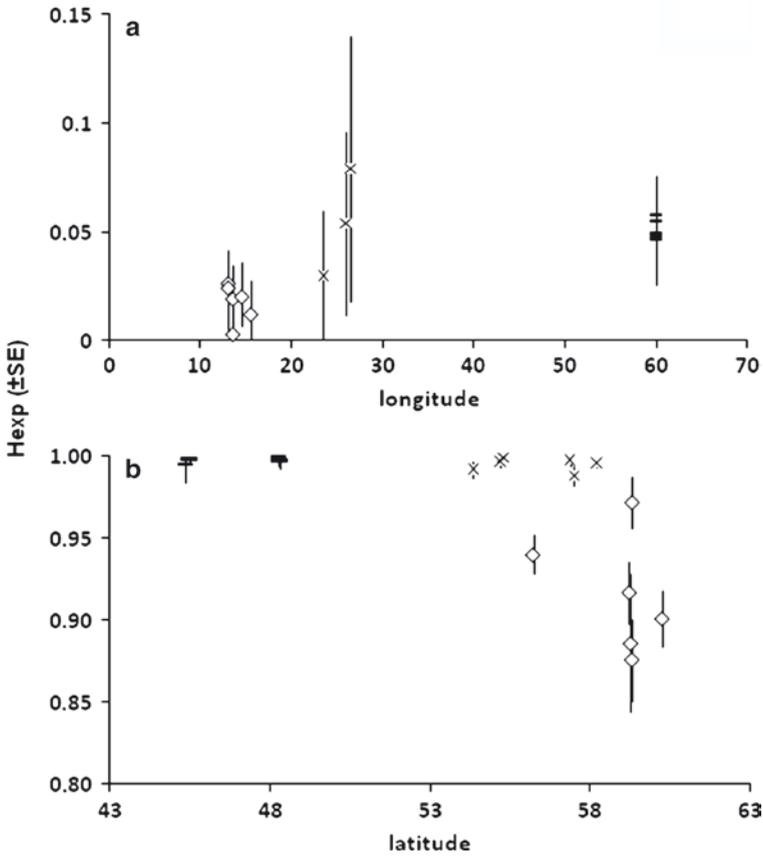
<i>C. hero</i>				
Regions	$P_{95\%}$ loci	Allelic richness	$H_{obs}$	$H_{exp}$
Isolated	16.7	1.20	0.03	0.03
Peripheral	20.0	1.43	0.09	0.12
Central	35.0	1.58	0.09	0.11
Differences among regions <sup>a</sup>				
Isolated/peripheral	n.s.	n.s.	**	*
Isolated/central	**	**	**	**
Peripheral/central	n.s.	n.s.	n.s.	n.s.
<i>C. arcania</i>				
Regions	$P_{95\%}$ loci	Allelic richness	$H_{obs}$	$H_{exp}$
Isolated	20.4	1.31	0.08	0.09
Peripheral	54.6	1.93	0.18	0.21
Central	45.8	2.06	0.18	0.20
Differences among regions				
Isolated/peripheral	**	***	***	***
Isolated/central	***	***	***	***
Peripheral/central	n.s.	n.s.	n.s.	n.s.

<sup>a</sup>n.s. = non significant, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

## 4 Selection Vs. Gene Flow

The adaptive traits representing overall wing size and shape followed the same pattern as the neutral markers. Individuals from the isolated region were significantly differentiated from the peripheral and central while the peripheral and central populations showed no signs of differentiation. If predicting that selection pressures are equal in the peripheral and isolated region, low or lacking levels of differentiation in both neutral and selected traits in the peripheral populations in combination with a significant differentiation in both marker types in the isolated populations suggest that gene swamping is causing the observed pattern. This is supported by the fact that the peripheral and isolated regions are found on the same latitude while the central populations are found on an order of magnitude more distant, suggesting that environmental conditions should be more equal in the peripheral regions rather than the peripheral and central ones.

However, I cannot from current data exclude that there are actually different selection pressures in the two peripheral regions. For at least two reasons both species experience a more fragmented landscape in Sweden than in the Baltic states. Firstly, Southern Sweden has a history of great changes in landscape configuration due to an intensive afforestation over the last century (Nilsson 1990), while the



**Fig. 2** Expected heterozygosity ( $\pm$  S.E.) for (a) 10 allozyme loci plotted against longitudinal position for 13 populations of *C. hero* and (b) 18 allozyme loci plotted against latitudinal position for 16 populations of *C. arcania*. Populations originate from three parts of the species' distributions; one core (Ural for *C. hero* and central Europe for *C. arcania*, population means indicated as straight line), one peripheral (Estonia for both, population means indicated as stars) and one isolated (Sweden for both, population means indicated as rombs)

**Table 2** Fixation index ( $F_{RT}$ ) and their significances among three regions of the two species *C. hero* and *C. arcania* within their distribution range

Regions	<i>C. hero</i>	<i>C. arcania</i>
Isolated/peripheral	0.26**	0.06***
Isolated/central	0.12**	0.04**
Peripheral/central	0.07**	0.01**

\*\* $p < 0.01$ , \*\*\* $p < 0.001$

See text for definition of regions

landscape structure has not changed considerably in, for example, Estonia during the same time period (Palang et al. 1998). Secondly, due to a significant decline in low intensive agriculture in Sweden over the past century (Dahlström et al. 2006), the proportion of semi-natural grasslands (primary habitats of *Coenonympha* species (Eliasson et al. 2005) is definitely lower in Sweden compared to the Baltic states. Both these processes have led to lower amounts of available habitat for the Swedish butterflies. As a consequence, both *Coenonympha* species may occur to a larger extent as locally isolated populations in Sweden, whereas the distribution approaches a continuous one in the peripheral Baltic (states) region.

## 5 Additional Threats to Peripheral Relict Populations

As described above, peripheral relict populations can also experience reduced levels of genetic variation which may reduce their potential to adapt to the environment and/or cope with stress (Bulmer 1971; Roff 1997). Therefore, even if peripheral relict populations are genetically unique their peripheral position can also cause an additional constraint to their survival capacity, where isolation may threaten their existence if the loss of variation adversely affects their current performance and ability to cope with changes in living conditions. In addition, if peripheral conditions select for less dispersive phenotypes, as appears to be the case in the examples described here, this will adversely affect their ability to react to improved climatic conditions. Even if a new habitat is created beyond the species current distribution range, the species may have lost their ability to track the newly created habitat if selection has removed good dispersers from the gene pool. If the differences observed are mirrored in flight performance, I could then expect that these peripheral populations can differ in reactivity to climate change (Haynes and Cronin 2006). Thus, individuals of the species found at the same range margin may differ in their reaction to changing climatic conditions and this should be taken into account when predicting the effects of climate change on species communities.

## 6 Conclusions

Irrespective of the exact processes involved in the populations described above, it shows that peripheral, relict populations of also widespread species can add valuable and unique genetic variation. However, these diversifying processes need to act in isolation as homogenizing gene flow from more central parts can significantly counteract both random processes like genetic drift as well as selective forces.

Despite a study setup that aimed to discriminate between the influence of gene flow and selection in these peripheral and isolated populations additional experiments are needed to draw convincing conclusions regarding the opposing alternatives of swamping gene flow and differing selection pressures. If landscape

structure is the cause for different phenotypes I would expect a correlation between habitat density and matrix configuration with wing sizes and shapes. If homogenizing gene flow, on the other hand, is causing the observed pattern I would expect the isolated phenotypes to be more successful than the central phenotypes in performance experiments in the peripheral region. These hypotheses can be tested and will add important insights into the evolution of peripheral populations and their reactivity to climate change. Further, marginal relict populations constitute ideal study objects of evolution in marginal habitats as they can be used to test how well theory and laboratory experiments reflects more complex natural systems (Kawecki, 2008).

**Acknowledgements** I thank all co authors and collaborators involved in various parts of the projects. Thanks to Atte Komenen, Ane Laugen and Thomas Schmitt for valuable comments on earlier versions of this manuscript. Part of the data presented here originates from work that was done at the Department of Conservation Biology and Genetics of Uppsala University, Sweden, and at the Department of Biogeography of University of Trier, Germany.

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# Genetic Differentiation Between and Among Refugia

Gabriel Nève and Régine Verlaque

## Abstract

1. Since the Last Glacial Maximum, the distribution of terrestrial plant and animal taxa in Europe has shifted from their refugia, Northwards in latitude and upwards in altitude.
2. The populations from refugia, mainly the mountain massifs of S Europe (Pyrenees and Alps, and also the Carpathian and Balkan Mountains), harbor a higher genetic diversity than the populations of N Europe.
3. The isolation by distance effect between populations tends to decrease with the increasing distance from the glacial refugia.
4. In areas of admixture from colonization originating in different refugia the heterozygosity may be higher than near the refugia.
5. Numerous thermophilous species have survived in their Mediterranean refugia at low altitude (particularly in islands).
6. Diploid populations or taxa often remained restricted at mid and high altitudes in S Europe, while their polyploid relatives spread in the lowlands.
7. For cold-adapted species, the refugia are mainly located in the S European mountains, and sometimes in N Eurasia, such as in Andøya island or in Taymyr peninsula.
8. The relict populations retaining a high allelic diversity and the endemic diploid taxa are now key elements in the long term conservation.

## 1 Introduction

Species which are widely distributed do not generally form one panmictic population. The degree of genetic variability varies within the distribution range. The first to formally recognize and search for genetic variability was the Russian scientist

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Nicolaï Ivanovitch Vavilov (1885–1943),<sup>1</sup> who set the foundations of research on the origin of cereals (in particular wheat). He demonstrated that their diploid wild relatives, in their original area, had a much higher variability than their widely cultivated generally polyploid relatives (e.g., Vavilov 1950). These principles were later followed by biologists studying the intraspecific variation of wild plants and animals. This same general principle may be applied to glacial relicts, with the question of the identification of the original area for each concerned species.

Since Forbes (1846) we know that the distribution of plants and animals changes with time, in particular during the Quaternary climatic oscillations. Haeckel formally recognised this when he wrote:

As the glaciation encroached from Northern Europe towards our Alpine chains, the polar inhabitants retreating before it - gentian, saxifrage, polar foxes, and polar hares - must have peopled Germany, in fact all Central Europe. When the temperature again increased, only a portion of these Arctic inhabitants returned with the retreating ice to the Arctic zones. Another portion of them climbed up the mountains of the Alpine chain instead, and there found the cold climate suited to them. The problem is thus solved in a most simple manner (Haeckel 1876, Chap. 14, p. 367).

The ca. 100,000 year cycle of glacial – interglacial periods has had a major impact on the distribution of organisms in the North hemisphere, particularly in Europe because of the East–west orientation of its main mountain massifs (Pyrenees, Alps, Carpathians), leading to a corresponding range shifts (Huntley and Webb 1989). For many cold-adapted species, the latitudinal Northward migration was accompanied by an upward altitudinal migration. These species then ended up with disjunct distribution, often called boreo-mountain or arctic-alpine species, depending on the altitude/latitude of their distribution (de Lattin 1967; Udvardy 1969).

From a genetic point of view, the key factors influencing the genetic structure of the populations of the species with such disjunct distributions are the age of the discontinuity in the distribution and the population size within each of the different distribution patches. For most European species distributed in both Scandinavia and the S European mountains (mostly in the Alps and the Pyrenees), it is assumed that the populations now found in, e.g., the Alps and the ones found in Scandinavia became separated ca. 10,000 years ago, i.e., at the end of the Würm glaciation. However, other scenarios are possible; N Europe may have been colonized from a refugium in Siberia, while Alpine populations were separated from these at a previous colonization cycle (Hewitt 1999). Furthermore, numerous thermophilous species remained at low altitude in the Mediterranean area during glaciations; afterwards some of them remained there while others spread Northwards.

The aim of the present chapter is to briefly discuss: (1) what are the long term consequences of range shifts in terms of endemic and cytotype distributions, and (2) how range shifts influence the genetic structure of populations.

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<sup>1</sup>The date of death of N.I. Vavilov is usually given as 1942 (e.g. Harland 1954). Details of his tragic death became known only after the demise of the Soviet Union (Azmanov 2002) and its date is 25 January 1943.

## 2 Karyology as a Marker of Genetic Variation

Chromosomal data are key variables in the genetic makeup of many plants and animals (Dobigny et al. 2004; Mabble 2004). These data accumulated over the last 80 years constitute a valuable material to look for a general framework about the relict cytotype distribution and the genetic differentiation within and among the refugia.

### 2.1 Endemism in the Mediterranean Basin

The Mediterranean Basin is characterized by its floristic richness (25,000 species) and high rates of endemism (around 12,500 species, i.e., 50%), while there is an obvious South–North gradient of decreasing endemism in Europe (Favarger 1972). The ten Mediterranean hot-spots of plant biodiversity represent privileged endemism areas and the most important refugia: mainly mountains and islands (Médail and Quézel 1997; Médail and Diadema 2008). About two thirds of endemics are perennial taxa that show a clear ability to colonize harsh habitats (coasts, screes, cliffs, rocky places, etc.), at mid and high altitudes (Médail and Verlaque 1997; Verlaque et al. 1997).

The remaining third are lowland endemics and constitute a threatened patrimonial flora (Verlaque 1999). For example, the diploid *Lysimachia minoricensis* of Minorca is now extinct in situ and the plants cultivated in the botanical gardens do not show any genetic variation (Calero et al. 1999). Except in such an extreme case, it is not possible to establish a general pattern of genetic variation according to the restricted or widespread status of taxa (Gitzendanner and Soltis 2000, Thompson 2005). Many rare species show a lower diversity than more common relatives, as theoretically expected, but there are many reverse cases. In the diploid endemic *Cyclamen balearicum*, the threatened populations of S. France (Languedoc) are genetically more differentiated than the healthy and numerous populations of Balearic Islands (Affre et al. 1997). Life history traits (life-form, age, mode of formation etc.; cf. Stearns 1992) and causes of rarity of endemic taxa play a major role in explaining these phenomena (Gitzendanner and Soltis 2000). It should be borne in mind that endemism may have two opposite causes: a small area may be the relict of a former widespread range or the result of a recent speciation. In the latter case, the new taxa may remain restricted neo-endemics, despite some colonization capacities, if they become isolated on an island or at high altitude, or if they are ecologically very specialized in rare habitats, as is the rule for most of the endemics.

In this context, a fourfold classification has been suggested based on the analysis of endemic taxa characters compared to those of their closely related species (Favarger and Contandriopoulos 1961; summarized by Thompson 2005). Proportions of endemic classes calculated in several islands and mainland areas indicate a general Mediterranean framework (Verlaque et al. 1997). The paleo-endemics (relict species morphologically isolated from other taxa) are mainly thermophilous taxa located in the lowlands, and are mostly present on islands. The patro-endemics

(generally diploid cytotype progenitors of polyploid relative taxa) occur at mid to high altitudes and are more present in mainland areas than in islands. The schizo-endemics (vicariant taxa with the same chromosome number) are generally diploid taxa living at high altitude. Lastly, the apo- or neo-endemics (polyploid derived cytotypes, younger than its relatives) mainly occur at low and mid altitudes.

Such a pattern allows the identification of two distinct processes: (1) the “passive” or relictual endemism (including paleo- and patro-endemics) and (2) the “active” or recent endemism (including schizo- and apo-endemics). The relictual endemism matches the refugia, and amounts only to 32% of endemics in Corsica, and 28% in Provence and Balearic Islands (Verlaque et al. 1997). As far as the recent endemism is concerned, the schizo-endemics are dominant (49–55% in W Mediterranean) and can be investigated mainly with genetic markers.

## 2.2 Polyploidy (i.e. Genome Duplication) in Europe

The occurrence of polyploidy into a group generally corresponds to an expansion phase (Goldblatt 1980; Lewis 1980; Thompson and Lumaret 1992). Conversely to the pattern found in endemism, by analysis of some lowland native floras, Reese (1958) shows a significant South–North gradient of increasing polyploidy, from the S Mediterranean area to N Europe [Table 1]. Brochmann et al. (2004) confirm this phenomenon and sum up the main historical hypotheses. In Europe, the intensity of Quaternary climatic changes has highly favored this polyploidy, due to a combination of phenomena: (1) the successive movements of floras (Huntley and Webb 1989), (2) their intermingling leading to numerous hybrids, and (3) the recurrent colonization of large virgin areas by the neo-polyploids during periods of glacial retreat. To these ecological factors, one must add (4) the fundamental role of genes controlling the meiosis, which can induce 2n-gametes (i.e., diplogametes) production when exposed to temperature changes and other stresses (Lexer and Fay 2005; Wang et al. 2004; Ravi et al. 2008). Thus, the Arctic region, the most affected by the glaciations, was re-colonized by a young flora very rich in allopolyploid taxa with a high heterozygosity (Brochmann et al. 2004).

The colonization power of polyploids can be explained by their wide ecological amplitude and tolerance: they are much more efficient competitors than their diploid

**Table 1** Proportions of polyploid perennial taxa from the native lowland flora

Area	Percentages	Latitude
North Sahara, Algeria	42.3	31° N
East Czechoslovakia	56.2	50° N
Sjaelland, Denmark	57.2	56° N
North Sweden	65.5	66° N
Svalbard	78.2	79° N

From Reese (1958)

**Table 2** Proportions of diploid species in the perennial taxa from SE France (Provence)

Total native flora	42%
Endemic taxa	52%
Rare non endemic taxa	60%
Rare endemic taxa	70%

progenitors, particularly in unstable conditions (Stebbins 1971; Ehrendorfer 1980; Levin 1983; Thompson and Lumaret 1992; Lumaret et al. 1997, Bretagnolle et al. 1998). Thus in France, the maximum percentage of polyploids is found among alien invaders (77%, Verlaque et al. 2002). Conversely, in native flora the higher rates of diploids reaches 70% in rare endemic taxa (Table 2) and 75% in critically endangered segetal plants (Verlaque and Filosa 1997). There is a strong correlation between the abundance of taxa and their ploidy level: polyploids correspond to the most common taxa, while the rare or extinct species are mainly diploids (Hodgson 1987).

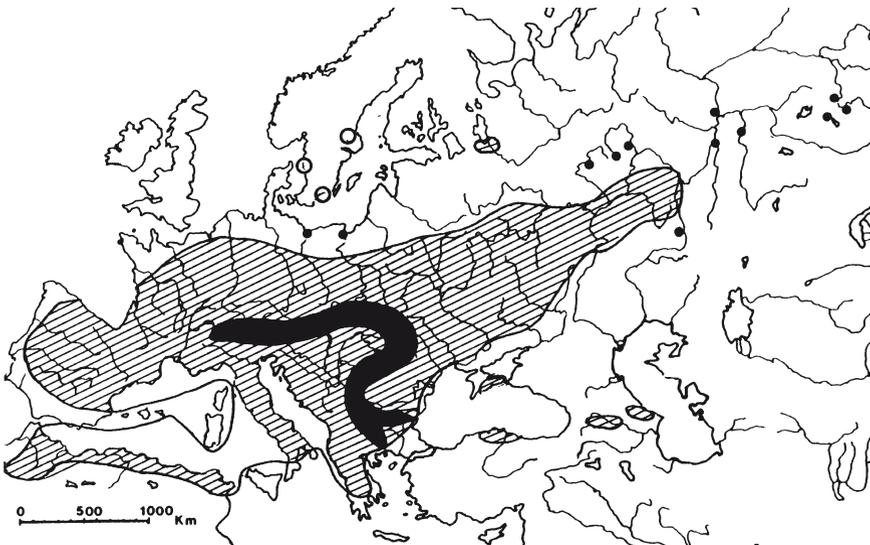
However, the study of polyploids is often very difficult because of their various possible origins: auto- or allo-polyploids from different populations (Levin 2002; Brochmann et al. 2004; Eidesen et al. 2007). A morphological and karyological approach is needed apart from DNA data in studies of plant phylogeny and “it seems advisable to investigate only the diploids (at least at first)” (Stace 2005). Polyploidy is generally a recurrent process followed by quick karyotype reshuffling, leading to a one-way process (Soltis and Soltis 1999; Crawford and Mort 2003). Genome duplication may also alter sexual reproduction and interspecific barriers; thus polyploids often become autogamous or apomictic (Richards 1997), or inter-hybridize (Breton-Sintès 1974; Gadella 1987). The intensive reticulation appears to be the main cause of phylogenetic incongruence because homogenization (of ITS sequences), via concerted evolution, may occur in various descendant lineages (Mansion et al. 2005). Lastly, the redundant structure of the genome in highly polyploid taxa often leads to a buffer effect against arising mutations.

During the glaciation maxima, the thermophilous species were pushed southward, and only the very dynamic taxa and cytotypes could (re)colonize successfully. In the Pteridophytes genus *Asplenium*, the 22 rupicolous diploidized species ( $2n=72$ ) remained mostly confined in the Mediterranean refugia after the Würm glaciation, only five diploid taxa colonized central Europe, the British Isles or Scandinavia. In contrast, the ten widespread taxa up to N Europe are polyploid ( $2n=144$ ). Furthermore, the diploid populations of *A. ruta-muraria* from Gorges du Verdon in SE France show the highest allelic variability known in ferns (Vogel et al. 1999). In Angiosperms, two examples could be cited: (1) the segetal EuroSiberian *Camelina sativa* includes one diploid relict population ( $2n=12$ ) in the SE France (Monts de Vaucluse, Verlaque and Filosa 1992), now critically endangered, one tetraploid in Bulgaria ( $2n=26$ ) and a majority of hexaploid populations ( $2n=40$ ) in Iceland, Poland, China and Russia; (2) the stenoMediterranean *Calicotome spinosa* is tetraploid, except in SE France (Var: Gapeau Valley) where a relict diploid cytotype still survives at low altitude (Aboucaya 1989, Fig. 1).

During the glaciations some diploid species already adapted to cold conditions persisted in refugia, especially in the S European mountains (Favarger 1967, 1972),



**Fig. 1** Distribution of *Calicotome spinosa*. Tetraploid populations ( $2n=48, 50, 52$ ) are found from Spain to Italy, while only one population in Var (France) was found to be diploid (*Diamond*:  $2n=24$ ). Distribution map compiled from Aboucaya (1989) and de Bolós and Vigo (1984)



**Fig. 2** Distribution of *Tanacetum corymbosum*. The diploid (subsp. *clusii*,  $2n=18$ , in black) is found only at 1,000–2,000 m altitude in the mountains from the Alps to the Rhodopes, while the tetraploid (subsp. *corymbosum*  $2n=36$ , hatched) is found in lowlands from Spain to Russia

while only polyploid cytotypes spread widely during the interglacial stages. In *Tanacetum corymbosum*, the diploid subsp. *clusii* is found from the Alps to the Rhodopes (1,000–2,000 m), while the tetraploid subsp. *corymbosum* is widely distributed in the lowlands from N Africa to Russia (Seidenbinder 1989; Verlaque and Contandriopoulos 1990; Fig. 2). In the orophyte *Ranunculus kuepferi* of Corsica and Alps, the diploid sexual relict subsp. *kuepferi* is restricted to the SW margin

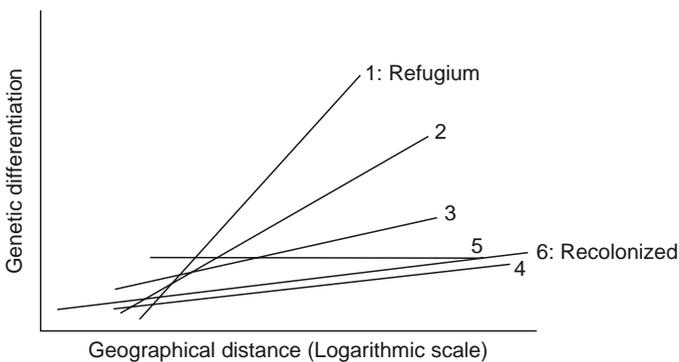
(Alpes-Maritimes, Var, Isère), whereas the tetraploid apomictic subsp. *orientalis* is widespread in the rest of the species range (Küpfer 1974).

### 3 Genetic Structure of Populations

The genetic structure of populations may be studied at different scales. The combination of genetic information from populations through a species' range leads to a knowledge of the spatial aspects of population genetics (Fig. 3). While investigating the population genetics of wild species, several indices may be used (Box 1). The most common for each population are the expected heterozygosity and the allelic richness. When the investigation concerns the structure of the populations, then  $F$  statistics and their derivatives are the most common indices (Wallis 1994), but the application of geostatistical techniques indicates that these traditional values might not be the best indicators of population differentiation (Joost et al. 2007, 2008).

Each of these genetic indices has its own objectives. The allelic richness is very sensitive to population bottlenecks and to population admixture. In the diploid beech tree *Fagus sylvatica* (W & C Europe, 0–2,000 m), the area of the highest heterozygosity in Europe corresponds to the area which was colonized between 4,000 and 2,000 years ago, which corresponds to the area of admixture of colonization fronts coming from different glacial refugia (Comps et al. 2001). On the other hand, the highest allelic richness is still found near refugia, i.e., the Balkan Peninsula and S Italy.

The process of colonization leads thus to a decrease of allelic richness, due to multiple foundation events, while colonization from different areas may lead to high heterozygosities, because of the admixture from refugia with different alleles (Widmer and Lexer 2001; Petit et al. 2001). For species which have colonized N Europe from a Southern refugium, their heterozygosity decreases with increasing



**Fig. 3** Isolation by distance effect in *Carabus solieri*. The slope depends on the time since colonization. The northernmost zones (4–6), i.e., the most recently colonized, show a more level slope than the areas close to the refugia (1), with a gradient of slopes for in between zones. Redrawn from Garnier et al. (2004)

## Box 1 Indices Used in Population Genetics

**H Expected Heterozygosity** Probability that two alleles sampled at random from a population are different. It is a function of the allelic frequencies. The expected heterozygosity is basically calculated for each locus, and a mean (or sometimes a median) value is presented for a number of loci. This index is particularly sensitive to bottlenecks having occurred in the population previously. This index is also called the Nei's gene diversity.

**Allelic Richness** The number of alleles per locus is a good index of diversity. It is highly sensitive to past population bottlenecks (Luikart et al. 1998) and investigated loci. As it depends on the number of sampled individuals, this index has to be normalised to a given sample size (El Mousadik and Petit 1996).

**Fixation Index**  $F_{ST}$  is an index of the way two or more populations are differentiated from each other. It depends on the difference of allele frequencies among the populations. It is usually estimated from allele frequencies in the concerned populations, as follows:

$$F_{ST} = (H_T - \bar{H}_s) / H_T,$$

with  $\bar{H}_s$  the mean expected heterozygosity of an individual in each population mating randomly, and  $H_T$  the expected heterozygosity of an individual in a total population mating randomly (e.g., Hartl and Clark 1989).

**Isolation by distance** The strength of any isolation by distance effect is measured as the slope of the regression of an index of genetic distance as a function of the geographic distance, both axes being expressed in logarithmic scale. The genetic distance is expressed either as  $F_{ST}$  or as  $F_{ST}/(1-F_{ST})$  for each pair of populations. The steeper the slope is, the stronger the isolation by distance effect would be.

**Neighborhood Size** Individuals often do not reproduce at the location of their parents. The genetic neighborhood has been defined as the area within which most of the individual will reproduce. Dispersal is the movement from the birth or parents' locations to the reproduction location. Wright (1943) defined the genetic neighborhood of an individual as the population of the area within the variance ( $\sigma^2$ ) of the dispersal distance. With  $d$  the density of individuals, the neighborhood size is given by  $N_e = 4\pi d\sigma^2$ . As the dispersal distance is usually difficult to measure, the neighborhood size is

(continued)

**Box 1** (continued)

often estimated from the differentiation between populations, using  $F_{ST}$  values estimated by pairs of populations to estimate the gene flow

$$M_{ST} \approx \frac{1}{4} \left( \frac{1}{F_{ST}} - 1 \right).$$

The y-intercept of the log-log plot of the estimated  $M_{ST}$  against geographic distance gives an approximation of the local effective population size (Slatkin 1993; example in Nève et al. 2008).

distances from their refugia. This has been called the “leading edge” effect, where the front (here North) part of the distribution moves, and the northernmost populations are the results of multiple colonization events (Hewitt 1993, 1996; Nichols and Hewitt 1994). The slope of the decrease of this heterozygosity depends on the rate of the loss of genetic diversity through the stepping stone colonization process.

During shifts of species range, the newly occupied area is the result of multiple colonization events. If this colonization is the result of rare events involving individuals only at the front edge of the distribution, a strong isolation by distance will follow together with a reduction of allelic diversity due to the repetitive colonization effect (Ibrahim et al. 1996). This resulted in a series of genetic hotspots distributed in the S Europe. Climate warming led to a gradual upward range shift, with little loss of genetic diversity, while the Northwards range shift involved long distance migration and multiple colonization bottleneck events. In the *Frangula alnus*, Eurosiberian diploid (0–1,400 m), the Iberian Peninsula and Turkey still harbor by far most of the genetic diversity. These probably correspond to the two main refugia for this species, and the colonization of the plains of central Europe was possible only by crossing severe barriers to dispersal (and hence gene flow) caused by the Pyrenees and the Black Sea (Hampe et al. 2003). In the arctic-alpine diploid species *Ranunculus glacialis* (1,700–4,000 m), populations from N Europe show a much lower genetic diversity than Alpine populations, as they resulted from a colonization from E Alps, where populations have retained a high genetic diversity (Schönswetter et al. 2003). In *Gentiana ligustica*, tetraploid endemic to the Ligurian Alps, the Shannon’s Diversity index, computed on AFLP fragments, decreases with increasing distance from putative Würm refugia (Diadema 2006).

The retreating range margin is also subject to important changes in the population structure. As the area becomes generally unsuitable to the species of interest, its local suitable habitat patches become scarce and far between. The populations

which used to be part of large networks of populations exchanging individuals with their neighbors become more isolated. These “trailing edge” populations are then reduced in their effective size, and are subject to drift, independently of other populations (Hampe and Petit 2005). The populations within the different mountain massifs of S Europe have been present in these areas often for several glacial cycles, resulting in levels of population exchanges to have occurred roughly according to the geographic distance. As at the continental scale the population exchanges have been rare, and as this process ended up being leveled over a very long time span (Slatkin 1993), the populations now found in the S European mountains often end up showing a very strong isolation by distance effect. If the North of Europe has been colonized from an Eastern refugium (“*Chorthippus* pattern,” Hewitt 1999), the populations of the mountains of S Europe may have been isolated from the Northern populations at least since the Riss glaciation (ca. 130–240 ky BP). In this case their long isolation resulted in a strong differentiation. The Capercaillie *Tetrao urogallus* of the Cordillera Cantabrica and the Pyrenees is an old lineage different from the rest of the species’ distribution, from the Alps and Scandinavia into Siberia (Duriez et al. 2007), which is the result of populations from the Iberian refugium not colonizing the lowlands North of the Pyrenees.

Within a given mountain massif, populations may also show a high differentiation, as they are separated by strong barriers such as valleys and rivers. If populations survived in nunataks during the last glaciation, the differentiation between these is the result of a pre-glacial isolation. In such cases, the populations now found at high altitudes of the Alps are the result of in situ survival during the Würm glaciation rather than post-glacial recolonization from peripheral areas. The perennial tetraploid cushion plant *Eritrichium nanum* most probably survived in at least three nunatak areas of the Alps (above 2,500 m), as these populations are the most variable (high number of private AFLP fragments) and the whole data set form three distinct clades (Stehlik et al. 2001). In other cases such as the artic-alpine diploid *Saxifraga oppositifolia*, genetics studies do not allow the identification of nunatak survival in the Alps, presumably because any surviving nunatak population would have been swamped by colonization from the main refugia (Holderegger et al. 2002). Sometimes a single pre-glacial population may end up being split: the tetraploid SW Alpine endemic *Senecio halleri* survived in two nunataks in the South part of its distribution, SW and NE of the Aosta valley, Italy. These two populations are not significantly different from each other, resulting probably from a single pre-glaciation colonization event (Bettin et al. 2007). Survival in and around mountain massifs during the last glaciation favoured the development of genetic differentiation between populations. In the widespread diploid *Arabis alpina*, the high among population variation in the mountains of S Europe (Alps, Tatra and Carpathians) suggests several refugia for this species, whereas all over the North of its distribution (Scandinavia, Iceland, Greenland and Newfoundland) it shows very low genetic variation, as these populations probably result from the recolonization from a single refugium closely following the retreating glaciers (Ehrich et al. 2007). Notably the populations in the East African mountains show high inter-populations variation, an evidence of their old age.

The carabid beetle *Carabus solieri* is an endemic species to the SW Alps near the Italian–French border. Garnier et al. (2004) sampled the whole distribution of this species, and performed genotypic identification of ten microsatellite loci in 1,080 individuals. Globally these populations show a strong isolation by distance, which is not surprising for a wingless species living in the mountain forests, in an area with many barriers to dispersal such as non-forested areas, roads, rivers etc. The 41 studied populations were grouped into three clusters, from South to North. Each cluster was then cut into two parts to have a series of six subclusters. Analysing these clusters separately for isolation by distance effect yielded a strong cline in the slope of the relationship between genetic distance and geographic distance. The slope is the steepest nearest to the refugium, and the most gradual in the recolonized area, i.e., northernmost set of populations (Fig. 3).

Other patterns of postglacial colonization have been described, from the refugia in central Asia or N Europe. The rare Alpine populations of the tetraploid *Carex atrofusca* originate from a central Asian refugium, while Siberia and Greenland populations form a sister clade (Schönswetter et al. 2006a). The diploid *Ranunculus pygmaeus* provides even a more extreme case as the Alpine populations probably originate from a refugium on the Taymyr Peninsula, East of the last glacial ice sheet (Schönswetter et al. 2006b). In Norway, the high nucleotide diversity of the populations of the root vole *Microtus oeconomus* from the island of Andøya strongly suggests in situ glacial survival for this species (Brunhoff et al. 2006). These examples show that the recolonization of the European ice sheet, after the last glacial maximum, took part from all directions, even if the contribution of the Southern refugia was prevalent (Fig. 4).



**Fig. 4** Map of Eurasia showing the Last Glacial Maximum (LGM; ca. 20 ky BP), with the main identified refugia of European taxa (LGM from Svendsen et al. 2004; Ivy-Ochs et al. 2008). Small glaciated areas in Mediterranean mountains (not shown here) are discussed in Hughes et al. (2006). *Filled circles*: main refugia; *Open circles*: nunatak refugia

## 4 Isolation by Distance

### 4.1 Differentiation Among Populations Within a Region

Differentiation among populations within each region is the result of the way individuals move within the region. All species are regionally distributed in an aggregative way: the species is found in its habitat, while it is missing or shows a low density outside its optimal habitats. Some species have a high dispersal power, and their populations are not differentiated within a region. This is the case of many migrating species, where dispersal between the birth location and the reproductive location is common. Many birds and insects (e.g., *Colias* and *Aglais urticae* butterflies; Vandewoestijne et al. 1999; Watt et al. 2003) follow this pattern. Wind pollinated plants also show genetic admixture in large areas, at least as far as the nuclear genome is concerned (e.g., *Quercus* spp., Petit et al. 2002).

The geographical scale of the isolation by distance effect varies with the dispersal scale of the species. In fixed organisms (i.e., plants), the scale of interest may be from a few meters (the distance between two individuals) to the extremes of a species distribution. Depending on the scale and on the dispersal capability, the relationship between geographic and genetic distance may or may not be significant. In the case of the Lycaenid butterfly *Euphilotes enoptes* there is no isolation by distance for distances of less than 30 km, but well in the range 30–400 km (Peterson 1995, 1996). The lack of correlation at small distances was related to the ecological heterogeneity at small scale, such as local topography, which has a profound impact on the species phenology. Isolation by distance at greater distances was attributed to a stepping stone dispersal process. Furthermore, the isolation by distance effect is mainly the result of long-distance migration events, while genetic neighborhood depends chiefly on the rate of movement between neighboring patches. In the butterfly *Procllossiana eunomia* neighborhood size is higher in the mountains than in the lowlands, while isolation by distance is higher in the mountains than in the lowlands (Nève et al. 2008). This shows that dispersal kernels may vary greatly between regions, and that population differentiation patterns may consequently also vary in different ways for short and long distances.

Differences in isolation by distance processes may also go the other way, with significance at the small scale distances of the spectrum, and no significance at a larger scale. In a series of 17 sampled sites for the diploid plant *Rhododendron ferrugineum* in the Alps and Pyrenees, no isolation by distance could be detected for site distances ranging from 4 to 1,000 km while, when a single site was studied in detail, the isolation by distance effect was significant for distances between 10 and 5000 m (Wolf et al. 2004). In this case, short distance isolation by distance was the result of short time scale bio-ecological factors, such as allogamy, pollination and seed dispersal, while the structure of populations on the Alps as a whole resulted from disequilibrium between dispersal and genetic drift, as very long distance dispersal does probably not follow a simple decreasing monotonous curve for this species.

## 4.2 *Differentiation Among Regions and Long Distance Gene Flow*

Species which were widely distributed during the Würm glaciation may end up having a now disjunct distribution. This may be the result of different processes: the populations of N Europe may have found themselves separated from the populations moving up the S European mountains or from a long distance colonization of newly available habitats such as islands in the high Arctic Ocean.

In arctic-alpine species, colonization may occur at very long distance. Isolated localities such as Bear Island and the Svalbard Archipelago had to be colonized over hundreds of kilometers of sea. Consequently, there is little isolation by distance in species showing such high colonization power. A recent study of nine species found on Svalbard showed that this archipelago was mainly colonized from NW Siberia (Alsos et al. 2007), rather than from the geographically closer Scandinavia and Greenland, because the main polar wind, from East, favored a colonization of high Arctic islands from Siberia (Coulson et al. 2002), and E Siberia (the Beringian area) was not covered by an ice sheet (Brochmann et al. 2004).

Among all the populations of the diploid mountain avens *Dryas octopetala*, Svalbard populations were most closely related to the Siberian ones. This arctic-alpine species displays an East–west differentiation, with five different groups in the Holarctic region (Skrede et al. 2006). The refugium of the European group probably laid in the tundra of C Europe during the Würm glaciation. As the ice retreated, this population split between the parts which migrated northwards to Scandinavia, and the populations which migrated up the mountains of the Alps. Among the European group, the Alpine populations show a much reduced variability ( $H < 0.077$ ) compared to the Arctic ones ( $0.101 < H < 0.144$ ). This unusual pattern is probably due to the widespread distribution of *D. octopetala* in C Europe during the Würm glaciation, which led to a latitudinal range shift involving several large refugia, while the colonization of the Alps involved fewer ones leading to a strong bottleneck effect.

The mating mode of organisms may increase the effect of isolation by distance. The perennial plant *Draba fladnizensis* mainly reproduces by selfing (as numerous arctic plants: Richards 1997). Morphologically, the individuals are homogenous throughout the arctic-alpine range, from N Europe to Alaska. Crossing experiments between specimens from different parts of the range mainly resulted in either no seed, or sterile seeds, while intra-population crossing yielded numerous viable seeds (Grundt et al. 2006). In this diploid species, the differentiation between populations proceeded rapidly, leading to cryptic speciation, even within a limited geographic area: Svalbard archipelago hosts at least three such sibling species. This speciation process (by minor translocation or mutation events, Brochmann et al. 2004) probably occurred in situ during the ca. 10,000 years since the height of the last glaciation was when Svalbard was completely covered by ice. A similar sibling speciation has been found in *Nigella* (Strid 1969) and *Erysimum* (Snogerup 1967) endemic taxa living in harsh habitats in the Aegean islands.

## 5 Conclusions

Processes of population differentiation had different consequences, according to the spatial and temporal scales at which they occurred. The differentiation between mountain massifs or between North and South Europe is a process due to the occurrence of long distance colonization or lack thereof. Some trends may be suggested: high arctic species tend to have a high colonization power, as their present range had to be efficiently colonized since the Würm glaciation, and also periodically during previous colonization cycles (Huntley and Webb 1989). For genetically spatially structured species, the focus should be on the most variable populations and on the ones which host many private alleles (Diadema et al. 2005). The knowledge of Evolutionary Significant Units (Crandall et al. 2000) taking into account both aspects, would be a valuable tool for conservation.

The postglacial colonization processes have favoured taxa with high vagility of propagules and a great competitive ability. In the latter respect, polyploids generally perform better than their diploid progenitors. However, the conservation of diploids is a key to the future evolution of the flora, their loss would be irreplaceable, as the families with the highest proportion of diploids are generally the ones with the most derived characters. Most of diploids have a better evolutionary potential than polyploids because minor modifications of their genome may alter the species more quickly and deeply. Lastly, harsh habitats, species rarity and endemism are key factors influencing the proportion of diploid in the native flora. Nowadays, disturbances caused by human activities have similar effects as glaciations, with the demise of many diploid stenotopic taxa, and the expansion of a few eurytopic polyploids, leading to an impoverishment and a banalization of the lowland flora.

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# Population Genetic Structure of Two Threatened Dragonfly Species (Odonata: Anisoptera) as Revealed by RAPD Analysis

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**Abstract** The riverine odonates *Macromia splendens* and *Oxygastra curtisii* were included in the European Habitats directive as taxa of special concern. Nevertheless, there is almost no genetic information about them. We assessed the genetic diversity and population structuring among several Northwest Iberian locations where these species occur. For this, we examined the genetic pattern revealed by RAPD markers in four locations of *M. splendens* and five locations of *O. curtisii*. The former showed strong population structuring, whereas gene flow between different river systems may be the reason for the lower structuring inferred for *O. curtisii*. Based on these results, we support the need of special management for *M. splendens* in Northwest Iberia.

## 1 Introduction

From the conservation point of view, the order Odonata is among the most thoroughly studied insect groups. This is because odonates are widely acknowledged as biological indicators (Foote and Rice 2005; Rouquette and Thompson 2005). Accordingly, many Odonata (and Lepidoptera) are flag species for conservation in some countries. This chapter evaluates the genetic diversity and population structuring of two species of Odonata (Insecta) occurring in Northwest Iberia: *Macromia splendens* (Pictet 1843) and *Oxygastra curtisii* (Dale 1834). These species are classified as endangered by different administrations

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(Sahlén et al. 2004), but data on their genetic diversity and population structure supporting such a categorization are lacking.

Conservation genetics of insects is a burgeoning scientific field (e.g., Legge et al. 1996; Gadeberg and Boomsma 1997; Rasplus et al. 2001; Jonsson et al. 2003; Lai and Pullin 2004). However, there are a few conservation genetic surveys specifically addressing dragonflies and damselflies (e.g., Watts et al. 2004, 2005a; 2006, 2007; Hadrys et al. 2006; Thompson and Watts 2006). As in most insect groups, the intensive development of highly variable molecular markers (e.g., Hadrys et al. 2005; Keat et al. 2005; Giere and Hadrys 2006; Lorenzo Carballa et al. 2007) and non-invasive techniques (Watts et al. 2005b) will soon provide a great amount of valuable data for nature conservation.

This work is the first population genetics survey on *M. splendens* and *O. curtisii*, two protected Odonata species. We aimed at (1) describing their genetic diversity and (2) estimating their degree of population structure. To achieve this, we screened the fine-scale genetic pattern showed by these species in several Northwest Iberian sampling sites. We used the Random Amplified Polymorphic DNA–Polymerase Chain Reaction (RAPD–PCR) markers.

## 1.1 The Studied Species

*M. splendens* is globally acknowledged as being vulnerable (Boudot 2006). This large anisopteran belonging to the Corduliidae family is the only European *Macromia* species. It occurs in Southeast France, Spain, and Portugal (Dommanget and Grand 1996) and inhabits highly localized biotopes (Cordero Rivera 2000). It appears to be distributed in three main groups of locations: France, Northwest Iberia, and Southwest Iberia. Other congeneric species are highly abundant and show more continuous distributions. Such a pattern led Lieftinck (1965) to suggest that *M. splendens* is a Tertiary relict. However, its rareness might also be due to the fact that its habitat has been quite inaccessible to researchers. Pollution and habitat destruction are the main problems for the conservation of this species (Sahlén et al. 2004; Ocharan et al. 2006, and references therein), also in NW Iberia (Cordero Rivera 2000).

*O. curtisii* is classified as Nearly Threatened by the IUCN Red List (Boudot et al. 2006). This member of the Corduliidae family mainly occurs in the Iberian Peninsula, and in the Western and Southern France. In addition, there are records from North and West Morocco (Jacquemin and Boudot 1999) as well as from Northern France, the Netherlands, Belgium, Western Germany, Switzerland, Northern and central Italy. This species is now extinct in Great Britain (d’Aguilar and Dommanget 1998). *O. curtisii* usually inhabits large rivers, preferring rapid waters. Interestingly, it is abundant in almost all Galician rivers (Northwest Spain, Azpilicueta Amorín et al. 2007). Therefore, a certain level of gene flow is expected.

## 2 Materials and Methods

### 2.1 Sampling

The two studied species (*M. splendens* and *O. curtisii*) were sampled between years 2000 and 2002. Sample sites and sizes (Table 1, Fig. 1) were selected according to the restricted distributional range of these taxa in NW Iberia. UTM coordinates were: Lérez 29TNH3501, Cabe 29TPH1201, Tea 29TNG4075, Albarellos 29TNG6492, Deza 29TNH6134, and Arnoia 29TPG0575. The minimum distance between two localities was 26.50 km (Tea–Lérez), whereas the maximum was 76.78 km (Arnoia–Deza). These five localities represented different ecosystems. Thus, sampling areas by rivers Lérez and Cabe represented slow and sunny stretches of deep rivers. Localities by rivers Tea and Deza represented smaller slow rivers. All river banks showed abundant vegetation. Interestingly, the vegetation type was Atlantic for Lérez, Tea, and Deza river basins, but Mediterranean for Cabe and Arnoia. Albarellos is a reservoir collecting water from river Avia. The total river length ranges from 50 km (Tea) to 84.5 km (Arnoia). Only river Lérez is not a tributary, running into the Atlantic Ocean. All sampled locations, except Albarellos and Arnoia, occur at Sites of Community Importance (SCI) within the Natura 2000 network.

Sampled populations of *O. curtisii* were large (about several thousand individuals per generation), whereas populations of *M. splendens* were smaller (hundreds of individuals, Cordero Rivera 2000; Cordero Rivera et al. 1999).

Adults were hand netted. We took one hind leg from each captured individual. Only captured males of *M. splendens* were sampled. Legs were stored in individual 1.5 mL tubes and frozen until analysis.

**Table 1** Sample sizes and genetic diversity obtained for two Odonata species in several NW Iberian locations

Species	Population	N	No. (and %) of polymorphic bands	Nei's gene diversity	Shannon's diversity index
<i>Macromia splendens</i>	Lérez	6	10 (40)	0.16±0.21*	0.24±0.30
	Cabe	11	17 (68)	0.26±0.20	0.39±0.29
	Tea	18	25 (100)	0.35±0.12	0.53±0.13
	Albarellos	4	10 (40)	0.16±0.21	0.24±0.30
	Overall	39	25 (100)	0.38±0.11	0.57±0.12
<i>Oxygastra curtisii</i>	Lérez	37	33 (100)	0.44±0.07	0.63±0.08
	Cabe	18	29 (87.9)	0.28±0.17	0.43±0.22
	Tea	20	30 (90.9)	0.33±0.16	0.49±0.22
	Deza	14	33 (100)	0.40±0.09	0.59±0.10
	Arnoia	10	26 (78.8)	0.32±0.19	0.46±0.27
	Overall	99	33 (100)	0.47±0.04	0.66±0.04

\*average ± standard deviation



**Fig. 1** Map of Galicia (NW Iberia) displaying the sampling locations for this genetic survey on two Odonata species: *Macromia splendens* and *Oxygastra curtisii*

## 2.2 DNA Extraction and RAPD Profiling

Whole genomic DNA was extracted by grinding the whole leg in liquid nitrogen. The obtained fine powder was homogenized in 500  $\mu$ L buffer (100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 0.5% SDS, and 50 mM DTT). The homogenate was incubated at 40°C for four hours in the presence of RNase (1 unit), and proteinase K (0.25 mg). Proteins were removed by extraction, first with phenol-chloroform and then with chloroform-isoamyl alcohol (2%). DNA was concentrated by ethanol precipitation (Sambrook et al. 1989). DNA samples were stored in TE 1X at a standardized concentration of 50 ng/ $\mu$ L.

RAPD-PCR markers have been successfully used to detect genetic polymorphism in other Odonata species (Andrés et al. 2000). We tested a total of 60 decameric random oligonucleotides (OPA01-OPA20, OPD01-OPD20, OPE01-OPE20, Operon Technologies, Inc.) in order to find polymorphism in a subsample of three individuals per species and from different localities. Eight of the primers (four per species, Table 2) proved to be highly polymorphic and were used to screen the entire collection.

Amplification reactions were performed in a final volume of 25  $\mu$ L containing 1  $\mu$ L 10X reaction buffer (Sigma), 1.5 mM MgCl<sub>2</sub>, 10 mM dNTPs, 2 pmol primer, 1 U Taq (DNA polymerase (Sigma)), and 50 ng genomic DNA. The thermocycler program used for amplification was: initial denaturation step (94°C for 5 min), 45 cycles (94°C for 1 min, 35°C for 1 min, 72°C for 2 min), final extension (72°C for 5 min), and cooling at 4°C. A negative control was always included to monitor any

**Table 2** RAPD primers (Operon Technologies Inc.) and summary of RAPD markers obtained for each studied species

Primer name	Primer sequence (5'-3')	<i>Macromia splendens</i>	<i>Oxygastra curtisii</i>
OPA-01	CAGGCCCTTC	12, 50–1,000, 6*	–
OPA-02	TGCCGAGCTG	15, 50–750, 7	–
OPA-04	AATCGGGCTG	11, 50–750, 8	–
OPA-08	GTGACGTAGG	–	13, 50–750, 5
OPC-09	CTCACCGTCC	–	17, 50–750, 11
OPA-09	GGGTAACGCC	10, 50–1,000, 4	–
OPE-11	GAGTCTCAGG	–	9, 50–1,000, 6
OPD-01	ACCGCGAAGG	–	10, 150–500, 11

\*The three values provided account for (1) number of scored bands, (2) size range of the marker (in base pairs), and (3) number of polymorphic bands (shared by <99% individuals)

possible DNA contamination. Fragments were resolved on 1.5% agarose gels and sized against a one Kilobase ladder (Sigma). Gels were run for 1.75 h at 120 V/400 A. DNA bands, stained with ethidium bromide, were visualized under UV light and photographed. All amplifications were repeated twice to prove the reproducibility of the RAPD-PCR bands. Only well-amplified and reproducible bands were considered for further analyses. RAPD bands were scored as present (1) or absent (0), and a binary matrix of different RAPD phenotypes was so assembled. RAPD bands shared by 99% or more individuals of the same species across the whole dataset were regarded as monomorphic and excluded from genetic analyses.

### 2.3 Statistical Analyses

We used three estimators to assess genetic diversity: (1) percentage of polymorphic loci ( $P$ ), (2) unbiased Nei's gene diversity for dominant markers, and (3) Shannon's index, which does not assume Hardy-Weinberg equilibrium. We used TFPGA v. 1.3 (Miller 1997) to calculate  $P$  and Nei's genetic diversity, whereas Shannon's index was calculated using POPGENE v. 1.32 (Yeh and Boyle 1997).

We assessed the extent of genetic structuring for each species using Weir and Cockerham's (1984) unbiased estimator,  $\theta$ , and its 95% confidence intervals (10,000 bootstrap iterations). We performed exact tests for population differentiation (Raymond and Rousset, 1995). These two analyses were calculated using TFPGA v. 1.3 (Miller 1997). The parameters of the Markov Chain Monte Carlo approach were: 1,000 dememorization steps, 10 batches, and 2,000 permutations per batch.

We tested for evidence of population subdivision using an analysis of molecular variance (AMOVA) as implemented in ARLEQUIN v.3.01 (Excoffier et al. 2005). We also used ARLEQUIN to test the genetic differentiation between pairs of populations using  $F_{ST}$  analogs. Statistical significance of covariance components (and

$\Phi$ -statistics) from AMOVAs and pairwise  $\Phi_{ST}$ 's was determined on the basis of distribution of values obtained from 10,000 permutations of data.

We used the Mantel test, as implemented in TFGPA, to estimate the correlation between genetic and geographic distances. A UPGMA tree of populations was constructed with TFGPA, using Nei's unbiased genetic distance. Branch support was estimated by bootstrapping across loci 10,000 times.

An individual-based rather than population-level analysis may provide a more accurate view of the current gene flow. This is because standard approaches like measures of population differentiation by  $F_{ST}$  rely on equilibrium assumptions and may, therefore, not be applicable to situations where equilibrium has not yet been attained. We used the program STRUCTURE 2.1 (Pritchard et al. 2000; Falush et al. 2003) for model-based Bayesian clustering of individuals without using prior population definitions. Following Pritchard et al. (2000), this approach was followed only for data not fitting the isolation-by-distance model (i.e., *M. splendens*). Simulations were run under standard settings in the no-admixture model, assuming correlated allele frequencies, without considering the population of origin of the samples. We used a burn-in of 250,000 iterations followed by 1,000,000 iterations for parameter estimation. Each simulation was run 10 times, exploring values for K (the total number of clusters to be constructed in a given simulation) ranging from one to four. To select the most appropriate number of groups, we followed Evanno et al. (2005).

### 3 Results

We obtained a total of 25 (*M. splendens*) and 33 (*O. curtisi*) polymorphic bands (Tables 1 and 2). Every sample produced a different haplotype. The average gene diversity values ranged from 0.38 in *M. splendens* to 0.47 in *O. curtisi*. (Shannon's index = 0.57 and 0.66, respectively). At a population level, both gene diversity and Shannon's index were lowest for *M. splendens* from Lézé/Albarellos and highest for *O. curtisi* from Lézé (Table 1). Sample size was positively correlated with both Nei's genetic diversity and Shannon's index (Pearson's  $r=0.81$ , and 0.80, respectively). The degree of population differentiation, as revealed by significant  $\Phi_{ST}$  values, varied from moderate (0.12, *O. curtisi* Lézé-Deza) to high (0.48, *M. splendens* Lézé-Cabe) (Tables 3 and 4). Within-population variation accounted for most of the total variation in both species ( $P<0.001$ , Table 5), regardless of grouping criteria such as ecosystem or phylogeny.

#### 3.1 *M. splendens*

We screened the genetic diversity at four Northwest Iberian locations, i.e., three rivers and one reservoir (Albarellos). The most diverse locality was Tea (Table 2). Overall, most of the variation (71.39%) was due to within-population genetic diversity

**Table 3** Matrix of population pairwise  $\Phi_{ST}$ -values obtained for *Macromia splendens*

<i>M. splendens</i>	Lérez	Cabe	Tea	Albarellos
Lérez	–	***	***	NS
Cabe	<b>0.48</b>	–	**	***
Tea	<b>0.31</b>	<b>0.13</b>	–	NS
Albarellos	0.37	0.47	0.25	–

Data in bold remained significant after sequential Bonferroni correction (*P*-values estimated after 10,000 permutations). Above diagonal: combined probabilities for each pairwise population differentiation (exact differentiation test). \* *P*>0.05, \*\* *P*<0.01, \*\*\* *P*<0.001

NS non-significant

**Table 4** Matrix of population pairwise  $\Phi_{ST}$ -values obtained for *Oxygastra curtisii*

<i>O. curtisii</i>	Lérez	Cabe	Tea	Deza	Arnoia
Lérez	–	***	***	***	***
Cabe	<b>0.21</b>	–	***	***	***
Tea	<b>0.15</b>	<b>0.40</b>	–	***	***
Deza	<b>0.12</b>	<b>0.31</b>	<b>0.24</b>	–	***
Arnoia	<b>0.16</b>	<b>0.21</b>	<b>0.34</b>	<b>0.26</b>	–

Data in bold remained significant after sequential Bonferroni correction (*P*-values estimated after 10,000 permutations). Above diagonal: combined probabilities for each pairwise population differentiation (exact differentiation test). \* *P*>0.05, \*\* *P*<0.01, \*\*\* *P*<0.001

NS Non-significant

(Table 5). There was significant population structuring according to sampling location, as suggested by both the test of exact differentiation ( $\chi^2=213.77$ , d.f. = 50, *P*<0.001) and *F*-statistics ( $\theta=0.29$ , CI=(0.22–0.35)). Regarding population-wise comparisons, the extent of differentiation ranged from moderate (Tea-Cabe,  $\Phi_{ST}=0.13$ ) to strong (Lérez-Cabe,  $\Phi_{ST}=0.48$ ). Albarellos was the only locality not significantly differentiated from any other (Table 3). No grouping was significantly differentiated from AMOVA analysis (Table 5), although river Cabe clearly separated from the rest of samples, as revealed by the UPGMA tree (Fig. 2a). Geographic and genetic distances were not correlated (Mantel test, *r*=0.38, *P*=0.26). Results from no-admixture clustering with STRUCTURE 2.1 indicated that the most likely grouping of *Macromia* individuals was *K*=2. The first two sampled localities (Lérez and Albarellos) appeared as purely belonging to a first population (POP1) (white, Fig. 3). The third sampled site (Tea) contained (1) 50% of individuals directly assigned to a second population, POP2 (grey, Fig. 3), (2) 11% of individuals directly assigned to POP1, and (3) 39% of individuals whose genetic pattern was intermediate between POP1 and POP2, but with a marked trend towards POP2. The fourth sampled locality (Cabe) was almost totally composed of individuals belonging to POP2.

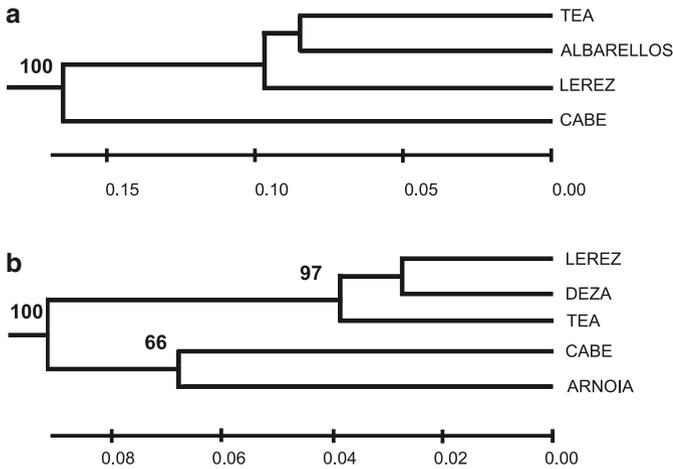
**Table 5** Analysis of molecular variance (AMOVA) across a range of putative population groupings

Species	Grouping criterion	Population grouping	Percentage of variation accounted for ( $\Phi$ )		
			Among groups ( $\Phi_{CT}$ )	Among populations within groups ( $\Phi_{SC}$ )	Within-populations ( $\Phi_{ST}$ )
<i>Macromia splendens</i>	–	All in one group	–	28.61 (0.29)***	71.39
	River basin	[Cabe/Tea/Albarellos] [Deza] [Lérez]	16.01 (0.16)	19.61 (0.23)***	64.38 (0.36)***
	Reservoir vs. river	[Albarellos] [all remaining POPs]	8.64 (0.08)	24.45 (0.27)***	66.91 (0.33)***
	STRUCTURE results	[Albarellos/Lérez] [Tea/Cabe]	21.87 (0.22)	13.51 (0.17)***	64.62 (0.35)***
	UPGMA tree	[Cabe] [all remaining POPs]	–4.69 (–0.05)	31.96 (0.30)***	72.73 (0.27)***
<i>Oxygastra curtisii</i>	–	All in one group	–	22.17 (0.22)***	77.83
	River basin	[Cabe/Tea/Arnoia] [Deza] [Lérez]	–15.05 (–0.15)	34.9 (0.30)***	80.17 (0.19)***
	UPGMA tree	[Cabe/Arnoia] [Tea/Deza/Lérez]	11.02 (0.11)*	15.13 (0.17)***	73.86 (0.26)***
	UPGMA tree	[Cabe/Arnoia] [Tea] [Deza/Lérez]	10.09 (0.10)**	13.56 (0.15)***	76.35 (0.24)***

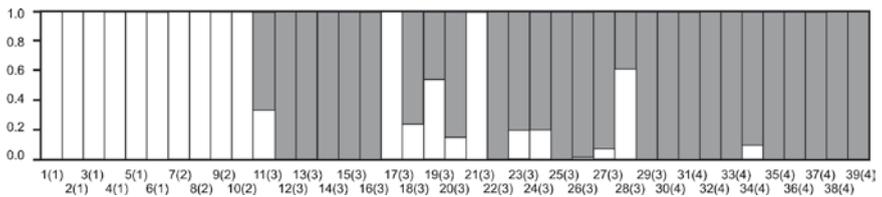
\*  $P=0.1$ , \*\*  $P=0.061$ , \*\*\*  $P<0.001$ . Values in italics correspond to  $\Phi_{ST}$

### 3.2 *O. curtisii*

We surveyed the genetic diversity of this species in five Northwest Iberian riverine localities. The most diverse locality was Lérez (Table 1). The within-population genetic diversity accounted for most of the variation (77.83%). There was significant population differentiation between sampling localities, as suggested by both the test of exact differentiation ( $\chi^2=446.69$ , d.f.=66,  $P<0.001$ ) and  $F$ -statistics ( $\theta=0.16$ , CI=(0.12–0.20)). Regarding population-wise comparisons, the extent of differentiation ranged from moderate (Lérez-Deza,  $\Phi_{ST}=0.12$ ) to strong (Tea-Cabe,  $\Phi_{ST}=0.40$ ) (Table 4). Geographic distance and genetic divergence were significantly



**Fig. 2** UPGMA dendrogram based on Nei’s unbiased genetic distance. Numbers at nodes are bootstrap values <50% (10,000 pseudoreplicates). (a) *Macromia splendens*, (b) *Oxygastra curtisii*



**Fig. 3** Results of STRUCTURE no-admixture clustering for *Macromia splendens*. Each individual is represented by a vertical column and the proportion of each of the two colors indicated the putative population they derive from. The most likely number of population ( $K=2$ ) was determined following Evanno et al. (2005). Numbers in the X axis identify each analyzed specimen ( $n=38$ ), giving its geographical origin in parentheses: (1) Lérez, (2) Albarellos, (3) Tea, and (4) Cabe

correlated in the area studied (Mantel test,  $r=0.61$ ,  $P<0.05$ ). The phylogenetic reconstruction showed two differentiated clades (Fig. 2b), in agreement with the geographic location.

## 4 Discussion

### 4.1 *M. splendens*

There was a high population structuring according to the sampling location. Regarding significant population-wise comparisons, the extent of differentiation ranged from moderate (Tea-Cabe) to strong (Lérez-Cabe). Lérez, Albarellos, and

Cabe were the poorest localities in terms of genetic diversity. An explanation for this might be that they are at the edge of the species' range. Gene flow may be more restricted between two groups of localities, as revealed by STRUCTURE: POP1 (Lérez-Albarellos) and POP2 (Tea-Cabe). This hypothesis requires further investigation using highly variable co-dominant markers.

Albarellos may have a high conservation value, as (1) it is the only studied locality not significantly differentiated from the others (Table 3) and (2) its geographic condition is intermediate between other sites inhabited by *M. splendens*. The reservoir Albarellos (336 ha, built in 1971) is the only sampled locality not included as SCI in the Natura-2000 network. Such an intermediate site may be important for a species with less dispersal and higher structuring than *O. curtisii*. Low dispersal in *M. splendens* is due to its ecological requirements rather than its actual dispersal ability, as anisopterans are usually good fliers. These facts point to the importance of preserving appropriate habitats and connectivity between them for *M. splendens*, a species less flexible in ecological demands, and clearly linked to remnants of autochthonous forests (Cordero Rivera 2006).

Seven out of the nine known Northwest Iberian populations of *M. splendens* were found in natural rivers. The remaining two sites were located in man-made hydroelectric reservoirs, where aquatic/riverine vegetation is lacking. Despite the negative effect of reservoirs on some insect groups due to habitat destruction (Turner 2007) and fragmentation (Watanabe and Omura 2007), it will be interesting to determine whether Albarellos resulted from a recent colonization from other river basins (e.g., Tea) or descended from individuals previously present in the river.

## 4.2 *O. curtisii*

There was a significant population structuring according to the sampling location. Regarding population-wise comparisons, the extent of differentiation ranged from moderate (Lérez-Deza) to strong (Tea-Cabe). AMOVA results (Table 5) suggested the presence of phylogeographic structure in our data. Clustering of *O. curtisii* populations, according to the branching pattern showed by the UPGMA tree (Fig. 2b), yielded a nearly significant value ( $\Phi_{CT}=0.101$ ,  $P=0.06$ ). This putative structure is congruent with the isolation-by-distance pattern inferred through Mantel's test. Accordingly, *O. curtisii* appears more widely distributed across the region studied than *M. splendens*. The former shows farther dispersal than the latter (Azpilicueta Amorín et al. 2007).

Arnoia was the only sampled locality not classified as SCI. We recommend this locality to be assigned a certain level of protection. This is based on the fact that Arnoia ( $n=10$ ) showed higher genetic variability than Cabe ( $n=18$ ). Both populations are geographically and phylogenetically close (Figs. 1 and 2). However, gene flow between them is expected to be low (Table 3). It was somewhat surprising that the highest level of genetic diversity was found in Lérez (Table 1). This is because

Lérez is probably the studied location suffering the most harmful human activity, e.g., intensive forestry with eucalypt plantations all over the basin, leisure facilities, and small dams.

### 4.3 Final Remarks

Different species with contrasting ecological demands react rather differently on the same landscape pattern (e.g., Louy et al. 2007). Our results indicated a higher structure for *M. splendens* than for *O. curtisii*, which agrees with the different dispersal of both species. However, these results should be taken as preliminary, due to the small sample size of some localities. For instance, Lérez was the locality where *M. splendens* ( $n=6$ ) was the least genetically diverse, but the one with highest gene diversity and Shannon's index for *O. curtisii* ( $n=37$ ). According to Aagaard et al. (1998), sample size might also explain the higher structuring of the two studied species compared to other Odonata (also surveyed using RAPDs) at similar geographical scale. Andrés et al. (2000) determined a low degree of structuring for *Ischnura graellsii* ( $\theta=0.061$ ), whereas Andrés et al. (2002) found a moderate differentiation for *Ceriagrion tenellum* ( $\theta=0.142$ ).

Different types of molecular markers may differ in the level of population differentiation they reveal (Volis et al. 2005). Therefore, it would be inappropriate to compare the extent of population structuring (as revealed by *F* analogs) we obtained for *M. splendens* and *O. curtisii*, with none or very little structuring found in *Nehalennia irene* (microsatellites; Wong et al. 2003), *Calopteryx splendens* (AFLPs; Svensson et al. 2004), *Lestes viridis* (allozymes, De Block et al. 2005), or *Ischnura elegans* (AFLPs; Abbott et al. 2008). In addition, comparisons with other studies should also include the geographical scale. The implications of a high population structuring ( $F_{ST}=0.25-0.48$ ) at fine scale (0–26 km), as shown by *Lestes viridis* (allozymes, Geenen et al. 2000) and *Coenagrion mercuriale* (microsatellites, Watts et al. 2004, 2006), might mislead the reconstruction of those processes leading to differentiation of populations as ours separated by 26.5–76.78 km.

To conclude, the present study determined that *M. splendens* is likely to be strongly structured in NW Iberia, whereas gene flow between different river basins may be the reason for the lower differentiation inferred for *O. curtisii*. Based on these results, we agree with the need of special management for *M. splendens*. Estimates of population size and assessment of putative connectivity networks between localities are needed for a better comprehension of the conservation status of *O. curtisii*.

**Acknowledgements** M. Pimentel and one anonymous referee improved the earlier versions of the manuscript with their comments. R. Sánchez-Guillén kindly helped in the laboratory. This work is part of MAA's PhD thesis. D. Romero designed Fig. 1. Funding was provided by grants from Spanish Ministry of Science (BOS2001-3642) and Xunta de Galicia (PGIDT01MAM37101PR) to ACR. MV was financed by the *Isidro Parga Pondal* program and the University of A Coruna.

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# Review

## Molecular Methods: Blessing or Curse?

Aline Finger and Charlotte Klank

**Abstract** Conservation genetic studies make use of molecular methods to obtain valuable information which help optimizing management strategies especially for threatened species. This chapter presents an overview of different molecular markers (microsatellites, AFLPs, RFLPs, RAPDs, mtDNA, allozymes) and their applications in conservation and genetic studies. Microsatellites have shown to be, though expensive, currently the most popular genetic marker as the high degree of polymorphism is ideal to study small geographical scales of species. RFLPs, RAPDs and allozymes still represent useful markers for studies of both, small and larger geographical scales. Low degree of polymorphism, no detection of alleles and low reproducibility characterize some drawbacks. To examine phylogeography MtDNA seems to be the best choice.

### 1 Introduction

Conservation studies rely increasingly on molecular methods to evaluate species “status quo,” historical distributions (biogeographical traits), and to develop management strategies for the restoration of populations. Since the invention of the polymerase chain reaction in the late 1980s (Mullis et al. 1996), further possibilities have opened up to apply genetic tools for diverse biological methods (see Fig. 1). Zhang and Hewitt (2003) revealed that, among the 1,758 primary papers and primer notes published between 1994 and 2003 in the journal of *Molecular Ecology*, 29.8 and 42.5% were indexed with mitochondrial and microsatellite DNA markers, respectively. Nevertheless, many more genetic methods exist to answer different ecological and genetic questions. Scientists, unfamiliar with the most commonly used genetic methods in the “conservation genetics field,” tend to get lost in the different molecular techniques as each has its own advantages and disadvantages,

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and can be applied in diverse forms. However, all these molecular techniques aim to detect genetic variation and differences within species, populations, or even individuals. This is generally achieved by comparing special DNA sections “marked” by the individual genetic method.

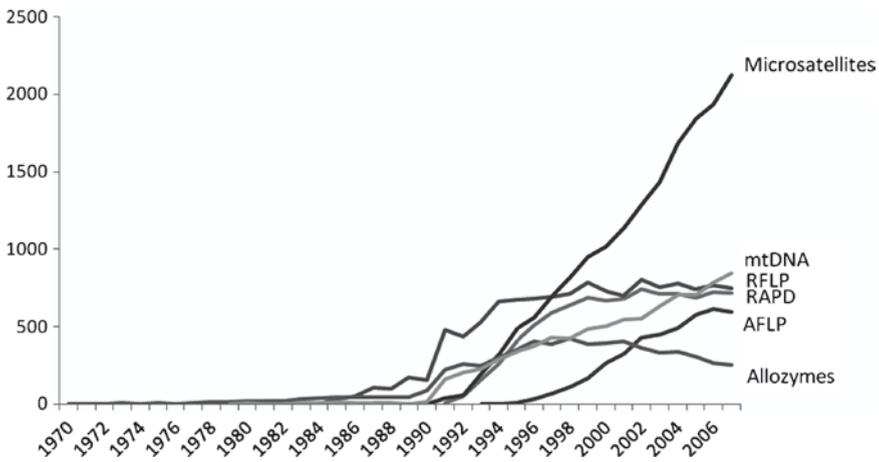
This chapter aims to present an overview of the most commonly used molecular methods during the last two decades (microsatellites, AFLPs, RFLPs, RAPDs, mtDNA, and allozymes) and the variety of ecological questions which can be answered with each method.

## 2 Molecular Markers and Their Application

### 2.1 *Microsatellites*

Microsatellites consist of short, tandemly repeated sequences of 1–6 base pairs within the nucleus of the cell (Palo et al. 1995; Ashley 1999). They have an elevated rate of mutation due to “slipped-strand mispairing” (Levinson and Gutman 1987; Palo et al. 1995; Eisen 1999), resulting in a high proportion of polymorphism even between closely related lines (Semagn et al. 2006 and references therein). Resulting variations (alleles) are scored through differing banding patterns. This marker is neutral to selection and is inherited co-dominantly as a standard Mendelian trait (Megléczy and Solignac 1998; Ashley 1999; Luikart and England 1999).

Microsatellites are the most commonly applied molecular marker in ecological research (Fig. 1). Their use in ecology and conservation studies is very broad and ranges from the identification of population genetic differentiation, demographic



**Fig. 1** Number of publications (selected biological subject) between 1970 and 2007 employing mtDNA, Allozymes, Microsatellites, RFLPs, RAPDs and AFLPs found via ISI web of knowledge

changes (e.g., bottlenecks, changes in effective population sizes ( $N_e$ ), genetic drift), to parentage analysis and the definition of management units (e.g., Jones and Ardren 2003; Wan et al. 2004; Leberg 2005; Olivieri et al. 2008; Orsini et al. 2008). Minimal preserved or dry samples are suitable for the microsatellite technique, allowing the analysis of ancient population patterns and genetic changes over time (Bruford and Wayne 1993; Megléc and Solignac 1998). However, microsatellites may have limited phylogenetic value due to homoplasy (Goldstein and Pollock 1997; Doyle et al. 1998; Selkoe and Toonen 2006). An informative review about microsatellites and their drawbacks can be found in the study by Selkoe and Toonen (2006).

## 2.2 *Mitochondrial DNA*

Mitochondrial DNA (mtDNA) is an extra-chromosomal genome in the cell mitochondria that resides outside of the nucleus, and is inherited from mother with no paternal contribution (Avisé 1991). The obtained PCR products are sequenced and banding patterns analyzed.

The theory of relatively constant mutation rates (molecular clock) (Lushai et al. 2003) is used to estimate time scales in which populations are split up, allowing the detection of, e.g., species dispersals and dispersal centers (Riddle and Honeycutt 1990; Rocha et al. 2008). Thus, genetic variation can be investigated on a broader geographical scale to unravel the historic (historical or recent) barriers to gene flow (Avisé 2000) and genetic basis of speciation and evolution of species and genera. Due to higher evolutionary rates of mtDNA relative to the nuclear genome (Wan et al. 2004 and references therein), this marker is preferred in constructing phylogenies and inferring evolutionary history, and is therefore ideal for within- and between-species comparisons (DeYoung and Honeycutt 2005; Behura 2006). Furthermore, mtDNA is believed to be the best tool for resolving taxonomic problems (Wan et al. 2004), identifying regions of endemism (Proudfoot et al. 2006), and Evolutionary Significant Units (ESUs) (Wan et al. 2004). Drawbacks of mtDNA analyses include hybridization, introgression, and incomplete lineage sorting. Moreover, mtDNA is of little use in investigating the recent loss of genetic variation and any individual-level events such as identity, individual dispersal, and mating systems (Wan et al. 2004).

## 2.3 *Allozyme*

Allozymes are enzyme variants due to allelic differences and can be visualized through protein electrophoresis. This technique was developed to quantify the genetic and geographic variation in wildlife populations, and it remains a cost-effective and straightforward method (Avisé 1994). Genetic variations caused by mutations are expressed as amino acid replacements due to changes in protein compositions, and are resolved as bands (alleles) on electrophoretic gels (DeYoung and Honeycutt 2005).

Allozymes have been important in plant biosystematics (see van der Bank et al. 2001) and are suitable for the detection of genetic variation within and between populations (Steiner and Joslyn 1979; Bartlett 1981; Loxdale et al. 1985). Due to a relatively low average heterozygosity, the application of this marker is suitable for geographically broader scales of extant species (van der Bank et al. 2001; Schmitt 2007). Even the analysis of parentage as single-locus polymorphism is sometimes possible with this marker (Chakraborty and Hedrick 1983; Meagher and Thompson 1986). Similarly, levels of hybridization, introgression, gene flow, and polyploidy can be studied. On the contrary, allozymes have limited phylogenetic value (e.g., Lowe et al. 2004 and references therein), represent phenotypic traits, and might be subject to selection. Another significant drawback is that only fresh material can be used to extract the proteins.

## 2.4 *Multilocus DNA Marker Systems*

Three of the commonly used multilocus DNA marker systems in evolutionary, taxonomic, ecological, phylogenetic, and genetic studies are RFLPs, RAPDs, and AFLPs (DeYoung and Honeycutt 2005; Behura 2006; Agarwal et al. 2008). All these markers generate banding patterns that are scored for variation. In all three markers, the detected variation is caused by either point mutation within recognition sequences as well as insertions and/or deletions between the recognition sites, which may lead to an underestimation of genetic variation (DeYoung and Honeycutt 2005). Since none of the described multilocus markers is specific to a certain target organism DNA, there is a risk of false variation generated by contaminations (Sunnucks 2000). Furthermore, dominance of some of the markers (RAPD and AFLP) does not allow for a detection of alleles.

Depending on the sampling strategy, these markers can cover a wide spatial range, allowing for a detailed fine scale analysis of population structure between individuals, especially with AFLPs (Meudt and Clarke 2007), up to taxonomically and spatially coarse studies (e.g., Despres et al. 2003). While RFLP, RAPD, or AFLP are unsuitable to estimate mutation rates or alike, and are thus inappropriate for temporal studies (i.e., evolutionary), they provide a detailed image of the present species or population genetic state.

### 2.4.1 **RFLP: Restriction Fragment Length Polymorphism**

Restriction fragment length polymorphisms (Botstein et al. 1980) are highly polymorphic, co-dominantly inherited markers based on the use of restriction enzymes which can be applied as single and multilocus probes with the banding patterns resulting from multilocus probes.

The technique generates highly reproducible banding patterns and is characterized by a high heritability (Lowe et al. 2004; Semagn et al. 2006; Agarwal et al.

2008). It is used in areas such as population and conservation genetics, diversity (e.g., Apostolidis et al. 2008), phylogenetics (e.g., Hu et al. 2008), linkage mapping (e.g., Tanksley et al. 1989), or cultivar identification (e.g., Busti et al. 2004), though their main application is within human genetics (Weising et al. 2005). As RFLPs require relatively large amounts of DNA, they have recently been replaced by PCR-RFLPs or AFLP analyses in most ecological studies.

#### **2.4.2 RAPD: Random Amplified Polymorphic DNA**

The dominant marker system RAPD, introduced by Williams et al. (1990), is based on arbitrarily amplifying DNA sequences during PCR, without prior knowledge of the organism sequence, using 10 nucleotide primers (Lowe et al. 2004, Weising et al. 2005). One of the main problems associated with RAPDs is their susceptibility to changes in reaction conditions leading to reproducibility problems (Jones et al. 1997; Agarwal et al. 2008; Assmann et al. 2007). Due to these problems, some peer-reviewed journals (e.g., *Molecular Ecology*) have recently changed their policy and publish RAPD data only in exceptional cases.

RAPDs have been used in many fields, among them are studies on population and conservation genetics (e.g., Kim et al. 2008), phylogenetics (e.g., Simmons et al. 2007), and linkage mapping (e.g., Sun et al. 2008).

#### **2.4.3 AFLP: Amplified Fragment Length Polymorphism**

AFLPs are dominant markers based on a combination of the RFLP and PCR techniques and were developed by Vos et al. (1995). Also, they do not require any previous sequence information and are based on the digestion of DNA by restriction enzymes and adapter ligation (resulting in universal primer binding sites), with two subsequent PCRs using specific primers. AFLPs are highly reproducible (Meudt and Clarke 2007; Agarwal et al. 2008) and primers can be combined to yield a large set of combinations, though this may also lead to a clustering of markers with certain restriction enzymes (Keim et al. 1997; Young et al. 1999; Saal and Wricke 2002).

AFLPs find wide application in studies on population genetics, diversity, and differentiation (e.g., Abbott et al. 2008; Tang et al. 2008), phylogenetics and taxonomy (e.g., Brouat et al. 2004; Schenk et al. 2008), hybridization (e.g., Volkova et al. 2008), linkage, gene, and genome mapping (e.g., Olmstead et al. 2008; Radoev et al. 2008), assignments (e.g., Yang et al. 2008) and kinship (e.g., Hardy et al. 2006). Although they are dominant markers, the large number of loci gives them a high statistical power (Meudt and Clarke 2007). Therefore, they are well suited for intraspecific studies (distinguishing between closely related individuals), where many loci are necessary (i.e., high genomic heterogeneity, low genetic variability), in polyploids, and in systems with hybridization occurring (Meudt and Clarke 2007).

**Table 1** Advantages, disadvantages and different features of microsatellites, Allozymes, mtDNA, RFLPs, RAPDs, and AFLPs

	Advantages	Disadvantages	Genomic abundance	DNA amount required	Polymorphism	Inheritance	Single loci
Micro-satellites	Medium costs (1)No sequence information needed (4) High reproducibility & reliability (4) Easy & safe protocol (4)Low costs (4, 8) Many enzyme systems (4) Suited for polyploids (4)	High start-up costs (1, 4) Species-specific primer pairs (4) PCR-based problems (5)	Medium (1, 2) – high (4)	Low (1, 2)	Medium (2) – high (1,4)	Co-dominant (1, 3)	Yes (3)
Allozymes	Easy & safe protocol (4)Low costs (4, 8) Many enzyme systems (4) Suited for polyploids (4)	Underestimation of genetic variation (4) Not sure whether neutral or not (10) Limited to extant populations (10) Only frozen or fresh samples (10)	–	–	Low (4)	Co-dominant (3)	Yes (3)
mtDNA	Constant mutation rates (11) Available primers Neutral and selective regions (6) Inherited maternally (7)	Little practical value for population genetic studies (8) Expensive	–	–	High (6)	Co-dominant (3)	Yes (3, 8)
RFLP	Detection of nucleotide DNA and organelle DNA polymorphisms (4) Phylogenetic analysis (4) High repeatability (1, 2, 4)	Labor intensive use (1, 4) High start-up costs (1, 4) Comparability between studies difficult (4)	High (1, 2)	High (1, 2, 4)	Medium (1, 2, 4)	Co-dominant (1, 3)	Yes (3)

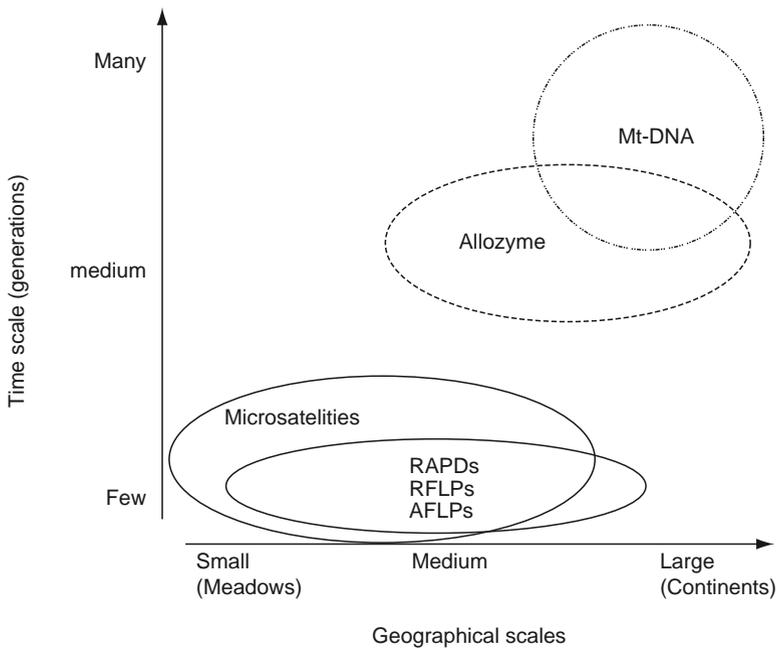
RAPD	Low costs (1, 4) Easy use (1, 4) No sequence information needed (4)	Low-intermediate reproducibility (1,2) Extensive criticism, see (4)	Very high (1, 2)	Low (1, 2)	Medium (2) – high (1)	Dominant (1, 3)	No (3)
AFLP	Medium costs (1)No sequence information needed (4) High reproducibility & reliability (4)	Initially difficult to set up (1)Technically demanding (4) Relatively high amounts of DNA needed (4)	Very high (1, 2)	Medium (1, 2) – high (4)	Medium (2) – very high (1,4)	Dominant (1, 3)	No (3)

Abbreviations: 1= Semagn et al. 2006; 2= Agarwal et al. 2008; 3= Sunnucks 2000; 4= Lowe et al. 2004; 5= Selkoe and Toonen 2006; 6= Wan et al. 2004; 7= Behura 2006; 8= Zhang and Hewitt 2003; 9= DeYoung and Honeycutt 2005; 10= van der Bank et al. 2001; 11= Lushai et al. 2003

### 3 Conclusions

The molecular methods presented in this chapter have question-related advantages and disadvantages (see Table 1). Before choosing a marker, it is essential to evaluate (1) which ecological question ought to be answered, (2) the spatial and temporal scales which ought to be explored, and (3) how exhaustive populations can be sampled (sample design; for detailed sample strategies, see Lowe et al. (2004), and (4) the available financial resources. Furthermore, the popularity of a specific marker might be important for the acceptance in a high ranking peer-reviewed journal, even though many markers could be suitable to answer the same question (Assmann et al. 2007).

In conclusion, we can say that microsatellites are currently one of the most popular genetic markers in ecological studies (see Fig. 1). Especially, the elevated rate of polymorphism is ideal to study small geographical scales of extant species (e.g., Finger et al. 2009). The use of allozyme markers is decreasing since several years (Fig. 1), although the low costs allow a high throughput for studying large geographical scales of extant species (Schmitt 2007). Similarly, AFLPs, RAPDs, and RFLPs can be used to study small to large geographical scales. These markers have their own drawbacks (AFLPs: no detection of alleles, RAPDs: low reproduc-



**Fig. 2** Different time and geographical scales applicable using mtDNA, Allozymes, Microsatellites, RFLPs, RAPDs, and AFLPs

ibility, RFLPs: high amount of DNA required, see Table 1). Finally, mtDNA is the best choice to study species history and phylogeography on broad geographical scales or deeper timescales (see Fig. 2).

**Acknowledgements** We would like to thank the professorship of Ecosystem Management, ETH (Jaboury Ghazoul) for funding. Furthermore, we are grateful for comments and corrections from Christopher Kaiser, Chris Kettle, Axel Hochkirch, and Andrea Pluess.

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# The EU Habitats Directive and the German Natura 2000 Network of Protected Areas as Tool for Implementing the Conservation of Relict Species

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**Abstract** This study analyses whether the Natura 2000 network of Sites of Community Importance (SCIs) is able to protect relict species in the taxonomic groups of higher plants, molluscs, dragonflies and damselflies as well as butterflies (only Rhopalocera) in Germany. Altogether, a total of 157 species from all groups are identified as relict species in Germany. Fourteen of these are included in Annexes II, IV or V of the Habitats Directive.

Most glacial relicts are well covered by an indirect protection regime of the European Union (EU) Habitats Directive as they occur in 46 of Annex I habitat types, and their occurrences are to a large extent covered by Natura 2000 sites (SCIs). For a few relict species and certain relict plant communities a gap remains in the EU protection regime, which can be filled by a national protection regime, for example, in nature reserves. The best way to protect local relict species is to include them in special management plans for their conservation.

## 1 Introduction

The protection of relict species is a prominent goal in nature conservation (Soulé 1986; Ås et al. 1992). These are taxa with only small recent occurrences and which now show only isolated distribution remaining from a once wider distribution.

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There may be very different reasons for their decline in the past. The species may be a relict of former stages of historical vegetation and landscape development resulting from past climate changes (glacial and post-glacial periods). Very often, the anthropogenic effects of cultural landscape development interfere or overlap with this, resulting in additional reduction of the distribution area, the density or fragmentation of patches or the population itself.

The scope of this paper is to focus mainly on the first category of relict species (which have developed mainly as a result of natural changes in abiotic conditions), as anthropogenic fragmentation and isolation has accelerated in recent decades and is at least partly documented in Red Data Books.

Most present-day relict species were widely distributed during the last glacial period, when their ecological requirements were well fulfilled; relict species with this type of distribution pattern are called climatic relicts or glacial relicts (e.g. Cox and Moore 2006). Today, these species are not well adapted to the prevailing climate conditions and are thus often very rare and localized, with the result that their occurrences are generally well documented. A prominent example of such glacial relict species is the dwarf birch (*Betula nana*) in bogs and fens in Germany. After the last glacial period, conditions were good for *B. nana* because cold-steppe vegetation covered the entire Central European landscape. Temperature increase resulted in more and more suboptimal conditions for *B. nana*, because other herbs and trees were more competitive and better adapted to the new conditions. *B. nana* was able to survive only in regions with low temperatures and low nutrient input where competition was reduced. Most glacial relicts, both plants and animals, follow this general pattern (Varga 1977). Often these species show arctic-alpine or boreo-alpine distribution patterns and are nowadays widely distributed in Northern Europe and/or remain isolated in cold areas such as the highest mountain regions or peatlands.

Thermophilic relicts represent a second type of relict species. These are the relicts from once warmer times during the Atlantic period, when thermophilic species were well adapted to warm and dry conditions. During this time, these species were widely distributed, but they later became isolated and restricted to favourable habitats due to colder temperatures and an increase of rainfall. A good example of a thermophilic relict is the Heath Bush-cricket *Gampsocleis glabra* in heathland in Northern Germany. In warm and dry years, Mediterranean species were able to expand to the North. When climate changes resulted in lower temperatures, these species were able to survive only in exposed habitats where higher temperatures are usually reached more quickly. This is often the case in stony and dry biotopes like dunes or rocks on Southern slopes. Areas with thermophilic relicts may often be found in the rain shadow of mountain ranges.

The goal of nature conservation is to conserve all these different types of relict species in their natural habitats as a witness of the natural conditions and developments in the past. Often it is quite clear that these species are not able to expand their ranges because current conditions are bad for them. In these often small populations with no contact to neighbour populations, evolution means that speciation and adaptation occur. This makes such populations very interesting objects of evolutionary research.

We have a responsibility to protect these species and their isolated populations. It is an important policy target to conserve a species' entire gene pool (BMU 2007; BFN 2008; Gruttke et al. 2004). The German government pointed out in its national strategy for biological diversity (BMU 2007) that the entire gene pool of species and populations has to be conserved, e.g. populations with local adaptations to regional microclimatic conditions. Fragmented populations often show significant changes in their character states, so if populations are separated for a long period from each other (several 1,000 years) with an interruption of gene flow, gene drift can mean that these populations change character states. The concept of Evolutionary Significant Units (ESUs) takes into consideration that such populations are on their way to an evolutionary unit of their own, leading to a different taxonomic status (Moritz 1994). For Germany, Gruttke et al. (2004) give a summary of current knowledge on this modern field of conservation biology, where glacial relicts are an important topic.

Nature conservation often means nature management. This involves the question of how relict species can be best protected. Since 1992, there has been an established, important and modern concept in nature conservation: The implementation of the EC Habitats Directive. The main goal of this Directive is to establish a network of Sites of Community Importance (SCI) for species listed in Annex II of the Directive. In Germany there are three biogeographic regions: Atlantic, Continental and Alpine (for further details see Balzer et al. 2008). One hundred and thirty-three plant and animal species as well as 91 habitat types are listed in Annexes II and I of the Habitats Directive, respectively. In Germany, a total of 4,622 SCI have been integrated into the EU network of protected areas "Natura 2000" for these species and habitat types. SCIs in Germany cover a terrestrial area of 3,313,083 ha equaling 9.3% of the territory. No scientific analysis has been performed to date to determine whether relict species are represented in the Natura 2000 network of protected sites in Germany. The current work gives an overview of whether, how and to what extent relict species are conserved by the EC Habitats Directive.

## 2 Method

A data analysis was carried out to determine whether the EU Habitats Directive provides sufficient protection for relict species in taxa of higher plants, molluscs, dragonflies and damselflies, and butterflies (Rhopalocera only) in Germany. For this purpose a database, containing for the most part glacial relict species, was set up based on studies by CLECOM (2002), De Lattin (1967), Holdhaus (1954), Kudrna (2002), Thorn (1960), Reisch (2001), Welk (2001) and Buchholz and Welk (2005). This database was modified for plants: i.e. *Gentiana clusii* was not accepted as a glacial relict species (populations in the Black Forest) as proposed by Thorn (1960) and Reisch (2001), because the species is not autochthonous there (Oberdorfer 1990). On the other hand, the database does not claim to be exhaustive for all potential glacial relicts with occurrences in Germany. Species on the edge of

their distribution area and endemics (other than relicts) in Germany were not included in the data analysis. Assessment was carried out on two levels:

1. Direct species protection was checked, i.e. whether the species is covered by Annex II (site protection within Natura 2000), Annex IV (strict species protection) or Annex V (limited protection of used or managed plants) of the Habitats Directive.
2. Protection of the habitat of the relict species was checked, i.e. whether the species is indirectly protected by the Natura 2000 sites: This can be assumed if the habitat of the species corresponds to a protected Annex I habitat type. We also assessed whether the occurrences of these glacial relicts are really covered by existing SCI and do not by chance lie outside the Natura 2000 network.

### 3 Results and Discussion

#### 3.1 *Glacial Relicts in Higher Plants and Their Protection by the Habitats Directive*

In total, 100 plant species were identified as glacial relicts in Germany (excluding *Rubus fruticosus* agg.). Most of the glacial relicts in higher plants belong to the alpine, pre-alpine or arctic-alpine distribution types (Table 1). Species that occur in Germany only in the Alps as part of their main distribution area but have relict populations outside of Germany only were not considered (six species, e.g. *Sibbaldia procumbens* in the Vosges or *Moehringia muscosa* in the Bohemian Forest). Six species are already extinct or presumed extinct in Germany, and another two species are extinct or presumed extinct in the isolated parts of their former natural distribution area outside the Alps (and the Alpine foreland), where relict populations remained until the last century (e.g. *Selaginella helvetica* formerly in the Thuringian Forest). A few other species are extinct in one or more discrete

**Table 1** Distribution of the glacial relicts of higher plants in Germany according to [www.floraweb.de](http://www.floraweb.de)

Area type	Number of species	Examples in Germany
Arctic-alpine	19	<i>Carex vaginata</i> , <i>Epilobium alsinifolium</i> , <i>Saxifraga stellaris</i>
Arctic (5), arctic-North (3), North (3), North-continental (1), North-prealpine (1)	13	<i>Carex paupercula</i> , <i>Pedicularis sceptrum-carolinum</i> , <i>Rubus chamaemorus</i>
Central Europe (2), central Europe-endemic (1)	3	<i>Pulsatilla alpina</i> ssp. <i>alba</i> , <i>Salix bicolor</i> , <i>Saxifraga oppositifolia</i> ssp. <i>amphibia</i>
Alpine	40	<i>Adenostyles alliariae</i> , <i>Carex sempervirens</i> , <i>Kerneria saxatilis</i>
Prealpine	25	<i>Anthriscus nitidus</i> , <i>Homogyne alpina</i> , <i>Soldanella montana</i>
Total	100	–

**Table 2** Relict plant species in Germany listed in the Habitats Directive

Species	Habitats Directive	Conservation status (National Report Art. 17, Hab. Dir.; Nationaler Bericht 2007)		
		Biogeographic region		
	Annex	Atlantic	Continental	Alpine
Marsh saxifrage <i>Saxifraga hirculus</i>	II, IV	Extinct in Germany		
Yellow gentian <i>Gentiana lutea</i>	V	–	Favourable	Favourable
Alpine clubmoss <i>Diphasiastrum alpinum</i>	V	–	Unfavourable - inadequate	Favourable
<i>Myosotis rehsteineri</i>	II, IV	–	Unfavourable - bad	–

parts of their former natural distribution area (e.g. *Gnaphalium norvegicum* in the Erzgebirge), but they still have recent relict populations in other parts of their distribution area in Germany. The 92 species that currently exist in relict populations in Germany were analysed in detail.

Direct protection by species annexes of the Habitats Directive is/was given only for four species (Table 2). This includes *Myosotis rehsteineri*, which is a glacial relict species according to Brackel (2001), but is not mentioned by the authors listed in the Methods section above. The species occurs at larger lakes at the Northwestern and Southern edges of the Alps and is classified as endemic in this region. Because the species is listed in Annexes II and IV of the Habitats Directive, we considered it in Table 2. *Saxifraga hirculus* is listed in Annex II; however, it is presumed extinct in its last known German locality in the Murnauer Moos, where Natura 2000 “came too late.” No other glacial relict is listed in Annex IV, and only two species (*Gentiana lutea* and *Diphasiastrum alpinum*) are covered by Annex V. Thus, the Habitats Directive affords very little direct species protection of relict species.

Table 3 shows the extent of indirect protection afforded by Annex I habitat types.

Only three glacial relicts (*Carex brunnescens*, *Epilobium alsinifolium* and *E. nutans*) grow outside Annex I habitat types and could not be attributed to one or more habitat types. These species are definitely not protected by the Habitats Directive.

As expected, the main occurrence of glacial relicts is concentrated on a few Annex I habitat types, which serve as refuges. These are hydrophilous tall herb fringe communities (Habitat type 6430), especially in their montane to subalpine subtypes of the class Betulo-Adenostyletea, which host 26 glacial relicts (28.3%); alpine and subalpine calcareous grasslands (habitat type 6170) which host 15 species (16.3%); calcareous rocky slopes with their chasmophytic vegetation (habitat type 8210) with 13 species (14.1%); and the calcareous and calcschist screes of the montane to alpine level (habitat type 8120) which host nine species (9.8%). On siliceous substrates, the situation is similar, with the siliceous alpine and boreal grasslands (habitat type 6150) hosting 20 glacial relicts (21.7%) and the species-rich *Nardus* grasslands in mountain areas (habitat type 6230) hosting 16 glacial relicts (17.4%). All these habitat types have in common the fact that during glacial

**Table 3** Coverage of relict species by Annex I habitat types (data base 92 glacial relict species)

Formation/group of habitat types	Number of species per habitat group	Annex I habitatcode	Habitat type	Number of species per habitat type	in %
Freshwater habitats	6	3220	Alpine rivers and the herbaceous vegetation along their banks	5	5.4
Heath and scrub formations	12	4060	Alpine and boreal heaths	5	5.4
		4070	Bushes with <i>Pinus mugo</i> and <i>Rhododendron hirsutum</i> (Mugo-Rhododendretum hirsuti)	8	8.7
Grassland and tall herb communities	65	6150	Siliceous alpine and boreal grasslands	20	21.7
		6170	Alpine and subalpine calcareous grasslands	15	16.3
		6210	Semi-natural dry grasslands and scrubland facies on calcareous substrates (Festuco-Brometalia)	6	6.5
		*6230	Species-rich <i>Nardus</i> grasslands, on silicious substrates in mountain areas (and submountain areas in Continental Europe)	16	17.4
		6410	Molinia meadows on calcareous, peaty or clayey-silt-laden soils (Molinion caeruleae)	3	3.3
		6430	Hydrophilous tall herb fringe communities of plains and of the montane to alpine levels	26	28.3
		6520	Mountain hay meadows	7	7.6
Bogs, transition mires and fens	16	*7110	Active raised bogs	5	5.4
		7120	Degraded raised bogs still capable of natural regeneration	3	3.3
		7140	Transition mires and quaking bogs	7	7.6
		7150	Depressions on peat substrates of the Rhynchosporion	1	1.1
		*7220	Petrifying springs with tufa formation (Cratoneurion)	4	4.3
		7230	Alkaline fens	6	6.5

(continued)

**Table 3** (continued)

Formation/group of habitat types	Number of species per habitat group	Annex I habitatcode	Habitat type	Number of species per habitat type	in %
Rock and scree habitats	24	8110	Siliceous scree of the mountains to snow levels ( <i>Androsacetalia alpinae</i> and <i>Galeopsietalia ladani</i> )	2	2.2
		8120	Calcareous and calcshist screes of the montane to alpine levels ( <i>Thlaspietea rotundifolii</i> )	9	9.8
		8150	Medio-European upland siliceous screes	1	1.1
		*8160	Medio-European calcareous scree of hill and montane levels	1	1.1
		8210	Calcareous rocky slopes with chasmophytic vegetation	13	14.1
		8220	Siliceous rocky slopes with chasmophytic vegetation	6	6.5
		8230	Siliceous rock with pioneer vegetation of the <i>Sedo-Scleranthion</i> or of the <i>Sedo albi-Veronicion dillenii</i>	2	2.2
		Forests	9	9140	Medio-European subalpine beech woods with <i>Acer</i> and <i>Rumex arifolius</i>
		*91D0	Bog woodland	2	2.2
		*91E0	Alluvial forests with <i>Alnus glutinosa</i> and <i>Fraxinus excelsior</i> ( <i>Alno-Padion</i> , <i>Alnion incanae</i> , <i>Salicion albae</i> )	1	1.1
		9410	Acidophilous <i>Picea</i> forests of the montane to alpine levels ( <i>Vaccinio-Piceetea</i> )	5	5.4

Double entries of glacial relicts occurring in more than one habitat types of Annex I are possible. \*indicates priority habitat type

and post-glacial times they had a wider distribution between the two main European ice shields in the North and in the Alps. This distribution then slowly diminished and became fragmented during the post-glacial warming-up. This development was aggravated and accelerated by anthropogenic influence which resulted in further

fragmentation and extinction in some of the refuges in the lower mountain ranges (e.g. Dörr 2000<sup>1</sup>). As the formations of mountain grasslands, tall herbs, rocky habitats and screes and, to a lesser degree, bogs are rich in glacial relicts and at the same time well represented in Annex I of the Habitats Directive, most of the species theoretically enjoy good indirect protection by the EU nature Directives. As this protection regime is valid only within accepted SCIs of the Natura 2000 network, it was necessary to verify coverage of the occurrences of glacial relicts in SCIs in order to assess the real protection regime. As not every occurrence could be checked individually, the results were classed in four different groups:

- (a) Occurrences (almost) completely within SCIs: 43 plant species
- (b) Sufficiently represented in SCIs, with a minor proportion of the occurrences outside: 47 species
- (c) Unclear, precise verification not possible: 1 species (*Salix myrtilloides*)
- (d) Not covered by SCIs: 1 species (*Minuartia stricta*).

Category (b) includes at least seven species, which are probably not or not sufficiently covered in every part of their fragmented occurrences, but the species are not in danger of extinction in Germany (e.g. *Hieracium bupleuroides* in the Franconian Alb). Furthermore, three species occur within Natura 2000 without, however, belonging to an Annex I habitat type, so they might not benefit from the EU protection regime.

Not only plant species but also whole plant communities (syntaxa) can be glacial relicts. These communities also need special conservation attention, not only because they are likely to host a number of glacial relicts in their fauna which has not yet been well studied. Rock-crevice communities of *Asplenietea trichomanis* are especially rich in relict species or paleo-endemics, as has been shown for *Kerneria saxatilis*, *Draba aozoides* and *Saxifraga paniculata* in the Swabian Alb by Wilmanns and Rupp (1966) and Wilmanns (1993). Spring formations of the class Montia-Cardaminetea, vegetation of snow-beds (*Salicetea herbacea*) and fens of the Scheuchzerio-Caricetea nigrae also have relict associations with the higher mountain ranges in central and Southern Germany. Examples of this are the Bryo-Philonotidetum seriatae (acid subalpine springs rich in mosses and *Saxifraga* species) on the Feldberg above 1,300 m altitude (Black Forest) with the nearest occurrences to the Alps, as well as the Bartsio-Caricetum fusca and possibly the Caricetum frigidae. A typical example of snow bed communities is the Nardo-Gnaphalietum supini on the Feldberg. Good examples of glacial relict communities are the Nardus grasslands of the higher mountains such as Leontodonto-Nardetum in the Black Forest, a vicariant association of the Violo-Nardetum (with *Pulsatilla alba* and *Viola lutea*) in the Vosges, the Lycopodio-Nardetum in the Bavarian and Bohemian forest and, finally, the Pulsatillo-Nardetum on the Brocken in the Harz mountains. Today, the subalpine tall herb communities of the Betulo-Adenostyletea have a nordic-alpine distribution with relicts in the lower mountain ranges, such as the Alnetum viridis, the

<sup>1</sup>This article includes some species as glacial relicts which were not listed as glacial relict species by Thorn (1960) and/or Reisch (2000) and were not considered in our study.

Adenostylo-Cicerbitetum and the Salicetum appendiculatae in the Black Forest, and the tall grass vegetation of the Sorbo-Calamagrostietum arundinaceae in both the Black Forest and the Vosges. These relict populations are of outstanding scientific and conservation interest, as their primary stands host species which were the ancestors of many grassland species of our cultivated meadows. The associations of the fluvial gravel deposits of the Salicion eleagni such as the Salici-Myricaritetum and the Salicetum eleagni with *Hippophae rhamnoides* ssp. *fluviatile* can also be regarded as relict vegetation, formerly widespread in the fluvioglacial plains of the ice ages. Some pine forests of the Erico-Pinion alliance, such as the Coronillo-Pinetum sylvestris, as a post-glacial relict of the warm period in the Swiss Jurassic (Richard 1972) and the Swabian Alb (Müller 1980) are examples of relict forests.

Of the relict syntaxa listed, most plant associations are covered by Annex I of the Habitats Directive, with two major exceptions: the acidic spring vegetation (Montio-Cardaminetea) and the Erico-Pinion relict pine forests. These merit special protection under national legislation in Germany, as long as they are not covered by the Habitats Directive.

### 3.2 Molluscs

A total of 342 species of non-marine molluscs were recorded from Germany in the CLECOM list (Falkner et al. 2001; CLECOM 2002) (the subterranean freshwater molluscs are not included here), of which 70 are freshwater and 236 terrestrial species. Twenty freshwater species are considered relicts (glacial or post-glacial isolated). The terrestrial molluscs are represented by 18 relicts.

The relict species include mountain molluscs as well as specialist species at low altitudes, for example in mires, fens and calcareous grasslands. Most relict populations suffer severely from bad environmental conditions. This situation has worsened in recent years because of an intensification of anthropogenic land use.

**Table 4** Relict molluscs species in Germany listed in the Habitats Directive

Species	Habitats Directive	Conservation status (National Report Art. 17, Hab. Dir.; Nationaler Bericht 2007)		
		Biogeographic region		
	Annex	Atlantic	Continental	Alpine
Snail, <i>Theodoxus transversalis</i>	II, IV	–	Unknown	–
Little Whirlpool Ramshorn Snail, <i>Anisus vorticulus</i>	II, IV	Unfavourable - bad	Unfavourable - bad	–
Freshwater Pearl Mussel, <i>Margaritifera margaritifera</i>	II, V	Unfavourable - bad	Unfavourable - bad	–
Geyer's whorl snail, <i>Vertigo geyeri</i>	II	–	Unfavourable - bad	Unfavourable - inadequate
Round-mouthed whorl snail, <i>Vertigo genesii</i>	II	Not evaluated 2007 because thought to be extinct; found again in 2008		

In Annexes II, IV and V of the EU Habitats Directive, 5 of these 18 relicts were represented (*Theodoxus transversalis*, *Anisus vorticulus*, *Margaritifera margaritifera*, *Vertigo geyeri*, *V. genesii*), all of which are represented in the German Natura 2000 network (Table 4). The remaining 13 mollusc relicts mainly live in the following habitat types of Annex I of the Habitats Directive:

- 3110 oligotrophic waters containing very few minerals of sandy plains (Littorelletalia uniflorae)
- 3130 oligotrophic to mesotrophic standing waters with vegetation of the Littorelletea uniflorae and/or of the Isoeto-Nanojuncetea
- 3140 hard oligo-mesotrophic waters with benthic vegetation of *Chara* spp.
- 3150 natural eutrophic lakes with Magnopotamion or Hydrocharition-type vegetation
- 3260 water courses of plain to montane levels with Ranunculion fluitantis and Callitricho-Batrachion vegetation
- 6210 semi-natural dry grasslands and scrubland facies on calcareous substrates (Festuco Brometalia)
- \*6240 sub-Pannonic steppe grassland
- 7140 transition mires and quaking bogs
- 7230 alkaline fens
- 8210 calcareous and calcshist screes of the montane to alpine levels (Thlaspietea rotundifolii)
- 9110 Luzulo-Fagetum beech forests
- 9130 Asperulo-Fagetum beech forests
- \*9180 Tilio-Acerion forests of slopes, screes and ravines
- \*91E0 alluvial forests with *Alnus glutinosa* and *Fraxinus excelsior* (Alno-Padion, Alnion incanae, Salicion alvae)
- 9410 acidophilous Picea forests of the montane to alpine levels (Vaccinio-Piceetea)

This assortment of habitat types and the German Natura 2000 network protects most of the 38 relict species. Only six species which prefer the potamic region of large streams, and *Vertigo lilljeborgii*, a glacial relict of acid sedge beds, are only partly included in the network. Not only the relict species but also other associated species – such as their specialized predators – should be protected. For example, some local populations of the Pyrenean shell crusher beetle *Carabus pyrenaicus* are so highly adapted to their prey, the relict mollusc *Arianta xatarti*, that these populations already show differences in mandible form compared to populations in the Western Pyrenees (Assmann et al. 2000).

### 3.3 Dragonflies and Damselflies

Eighty-one dragonflies and damselflies are known to occur in Germany, and two of them are listed as extinct. Only 7 of the 81 species were recognized in this study as relict species (cf. Sternberg 1998). *Coenagrion hylas* is extinct in

**Table 5** Relict dragonflies and damselflies species in Germany listed in the Habitats Directive

Species	Habitats Directive	Conservation Status (National Report Art. 17, Hab. Dir.; Nationaler Bericht 2007)		
		Biogeographic region		
	Annex	Atlantic	Continental	Alpine
Damselfly, <i>Coenagrion hylas</i>	II	Extinct in Germany		
Siberian Winter Damselfly, <i>Sympecma paedisca</i>	IV	Unfavourable - bad	Unfavourable - inadequate	Unknown

Germany (one of the two extinct species in Germany), and five of the remaining six species are specialists of dystrophic habitats. Two species are listed in the Annexes of the Habitats Directive (Table 5). Most of the breeding habitats of these five species (*Nehalennia speciosa*, *Aeshna caerulea*, *A. subarctica*, *Somatochlora alpestris*, *S. arctica*) belong to the following habitat types of the Habitats Directive:

- 7110 active raised bogs
- 7120 degraded raised bogs still capable of natural regeneration
- 7140 transition mires and quaking bogs
- 3160 natural dystrophic lakes and ponds.

Although many of the populations and habitats of the relict species are protected by the network of Natura 2000, their conservation status is not good. The main reason is the unfavourable conservation status of their habitat types. In the Atlantic and the Continental biogeographic regions, all four habitat types are considered to have an unfavourable conservation status. Only in the Alpine biogeographic region is the conservation status mostly favourable. The essential threats to these low-nutrient habitats are the intake of nutrients and the exposure level of air pollutants (even by air), the lowering of ground water levels and recultivation of bogs. As a result, the typical poor nutrient water ecosystems and the water chemistry have to be safeguarded, and buffer zones should be created to minimize the inflow of nutrients from surrounding surfaces. The damselfly *Sympecma paedisca* is endangered in Europe and strongly protected by the EU Habitats Directive Annex IV. It is a relict with a Continental distribution. Beneath moorland habitats such as dystrophic waters more nutrient-rich standing waters were also colonized. Although there are no sites especially protected by the Habitats Directive for this species, many of the populations are found in protected areas in Germany. The conservation status of the species in the Atlantic and Continental region is unfavourable - bad and unfavourable - inadequate, respectively, and is unknown in the Alpine region. The number of populations of *S. paedisca* in Germany is decreasing severely, at least in Lower Saxony and Baden-Württemberg. Besides habitat degradation, climate change could also be one of the reasons for the decline (Ellwanger and Mauersberger 2003).

### 3.4 Butterflies

Currently, 188 butterfly species are established in Germany (Lepidoptera: Rhopalocera). Twelve species from two different groups of relict species were identified as relicts. Butterflies such as *Lycaena helle* represent species that are well adapted to cold-stenothermic conditions. Examples for species in Europe that are adapted to cold conditions, and thus defined as glacial relicts, can be found in the work of Varga (1977) and Weidemann (1995). Other species such as *Chazara briseis* are thermophilic relicts from warmer times.

Only one of these relict species in Germany is listed in Annex II of the Habitats Directive (Table 6). This means that relict species in Lepidoptera (Rhopalocera) are underrepresented in Annex II, because SCIs have only been established for one species in the network of Natura 2000 sites. For the other 11 species, it is not obligatory to integrate their populations in the network. In Annex IV another two species are listed (Table 6). These are protected by law, but no special sites (SCIs) need to be set up. Most of the relict species settle in habitat types listed in Annex I of the Habitats Directive, and SCIs should also be selected for those habitat types. Thus, there is a good chance of saving the locations of Germany's butterfly species with the protection of the Annex I habitat types.

All thermophilic relict species live primarily in the following habitat types of Annex I of the Habitats Directive:

- 5110 stable thermoxerophilic formations with *Buxus sempervirens* on rock slopes (Berberidion p.p.)
- 5130 *Juniperus communis* formations on heaths or calcareous grasslands
- \*6110 rupicolous calcareous or basophilic grasslands of the Alysso–Sedion albi
- \*6120 xeric sand calcareous grasslands
- 6210 semi-natural dry grasslands and scrubland facies on calcareous substrates (Festuco-Brometalia)
- \*6240 sub-pannonic steppe grasslands
- 8210 calcareous rocky slopes with chasmophytic vegetation
- 8220 siliceous rocky slopes with chasmophytic vegetation
- 8230 siliceous rock with pioneer vegetation of the Sedo-Scleranthion or of the Sedo albi-Veronicion dillenii.

**Table 6** Relict butterfly species in Germany listed in the Habitats Directive

Species	Habitats Directive	Conservation Status (National Report Art. 17, Hab. Dir.; Nationaler Bericht 2007)		
		Biogeographic region		
	Annex	Atlantic	Continental	Alpine
Scarce Heath, <i>Coenonympha hero</i>	IV	Unfavourable - bad	Unfavourable - bad	–
Violet Copper, <i>Lycaena helle</i>	II, IV	–	Unfavourable - inadequate	Unfavourable - bad
Apollo, <i>Parnassius Apollo</i>	IV	–	Unfavourable - bad	Favourable

No thermophilic relict species is listed in Annex II, and only one (*Parnassius apollo*) is listed in Annex IV. But all four thermophilic relict species in Germany can be found in the habitat types described below and also in 2310 (dry sand heaths with *Calluna* and *Genista*), 2320 (dry sand heaths with *Calluna* and *Empetrum nigrum*), 2330 (inland dunes with open *Corynephorus* and *Agrostis* grasslands) and 4030 (European dry heaths).

Eight cold-stenothermic relict species occur in Germany. One of these is listed in Annex II and IV (*Lycaena helle*) and one only in Annex IV (*Coenonympha hero*).

The cold-stenothermic relict species live primarily in the following habitat types of Annex I of the Habitats Directive:

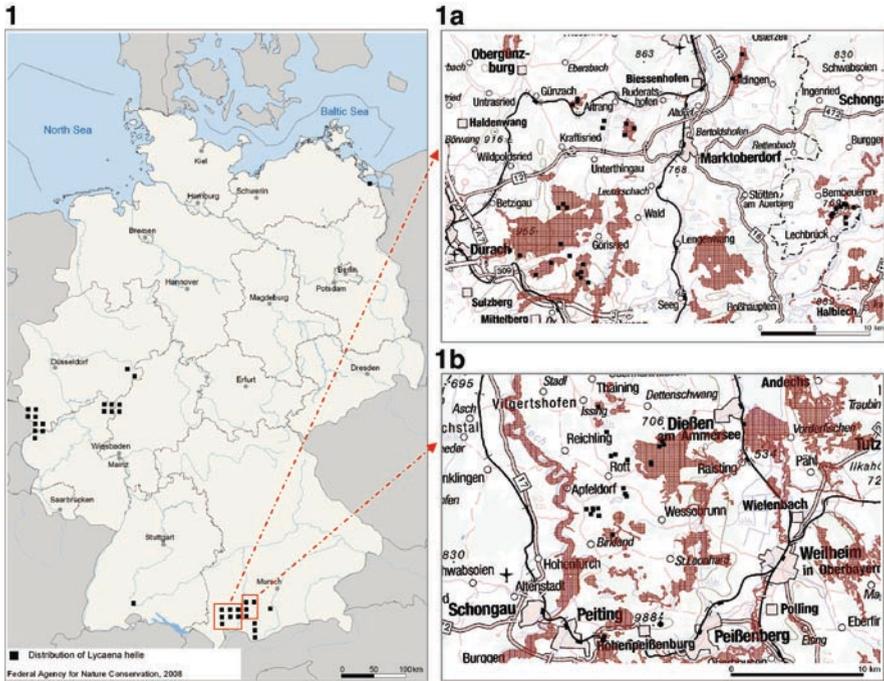
- 4010 Northern Atlantic wet heaths with *Erica tetralix*
- 4060 alpine and boreal heaths
- \*6230 species-rich *Nardus* grasslands, on silicious substrates in mountain areas (and submountain areas in Continental Europe)
- 6410 *Molinia* meadows on calcareous, peaty or clayey-silt-laden soils (Molinion caeruleae)
- 6430 hydrophilous tall herb fringe communities of plains and of the montane to alpine levels; important for feeding habitats)
- \*7110 active raised bogs
- 7120 degraded raised bogs still capable of natural regeneration
- 7140 transition mires and quaking bogs
- 7150 depressions on peat substrates of the Rhynchosporion
- 7230 alkaline fens
- \*91D0 bog woodland.

The locations of most of the cold-stenothermic relict species are protected through this assortment of habitat types and with the German Natura 2000 network.

### 3.4.1 The Violet Copper (*Lycaena helle*) in Germany

The distribution area of the Violet Copper ranges from Scandinavia to Russia. It is also distributed over Central Europe and the Alps, and reaches East into Siberia (Region Amur) (Weidemann 1995). The species belongs to the Northern-boreal distribution type and is classified as a cold-stenothermic relict species in Germany.

The distribution map shows a high level of fragmentation in Germany (Fig. 1): In the German low mountain ranges, the species is present only in peninsulas in the Westerwald, Eifel, Rothaargebirge and in an isolated area in the Northern German lowlands near the river Oder in Eastern Germany. The best areas are in the prealpine lowlands of the Alps, where this species shows the highest abundances in Germany and the largest coherent area without interruptions. Nearly all localities in Germany are found in the Continental biogeographic region, with the exception of one population in the German Alpine biogeographic region (near Garmisch-Partenkirchen). The conservation status in the German Continental biogeographic region was assessed as unfavourable - inadequate in the German National Report (Art. 17 Habitats Directive)



**Fig. 1** Distribution of *Lycaena helle* (Source: German National Report according to Art. 17 Hab. Dir.) (a, b) Occurrences of *Lycaena helle* (1992–2003, black quadrat) and the Sites of Community Importance (Data as of Dec. 2006, red areas) in Bavaria

in 2007 and in the Alpine biogeographic region as bad (red). Figure 1 shows the current distribution area in Germany.

Imagos of *Lycaena helle* can be found during only a short period from May to June and only in cool climates and wetlands with *Calthion* (also *Cirsietum rivularis* and *Chaerophyllo-Ranunculetum aconitifolii*), where *Polygonum bistorta* occurs. Habitat types with these associations are not well represented in Annex I of the Habitats Directive.

The Violet Copper was submitted to Annexes II and IV during the enlargement of the European Union (EU) to the East in 2004 (Biewald and Nunner 2006). All member states with occurrences of such newly listed species in the Habitats Directive are responsible for their protection. However, the old member states were not urged to install SCIs for the species added to Annex II, but they do fall under the protection of already existing Natura 2000 sites. In Germany, no area has been especially nominated to date for Natura 2000 for *Lycaena helle*, so this species occurs only in a few SCIs where other protected species of Annex II also occur. Only four of the six federal states in Germany where *Lycaena helle* is found have so far completed their duty to update the Standard Data Forms indicating the presence of the species within their sites. Germany has to ensure protection of *Lycaena helle* as it is listed in Annexes II and IV of the Habitats Directive, so a Natura 2000

network will need to be implemented for the protection of this species in the next few years. Figure 1a, b shows the problem that only few habitats of *Lycaena helle* are protected so far in Germany. The best areas should be integrated in the list of SCIs as soon as possible.

Figure 1a shows a small region of Bavaria to the East of Durach, where most areas with populations of the Violet Copper can be found in Natura 2000 sites. In other regions in Bavaria, for example in the West near Lake Ammersee, most remaining populations are located outside Natura 2000 sites (Fig. 1b). Because most settled areas in Western Germany are not represented by Annex I habitat types, there are not enough sites represented in the network of SCIs.

Only one relict butterfly species is listed in Annex II of the Habitats Directive in Germany. Another two species are listed in Annex IV. Most populations of all identified 12 relict species of day-flying butterflies in Germany live in Annex I habitats, so protection for these species is possible in their habitats. The habitats of the Violet Copper as a new species in Annex II are not well represented in the Annex I habitat types. The conservation status of the species in the Continental biogeographic region is unfavourable - inadequate, and in the Alpine biogeographic region it is unfavourable - bad. The specific situation of this species will obviously need additional SCI proposals or enlargement of existing SCIs to fulfil the requirements of the Habitats Directive, and this might be the only means of securing a better conservation status in future. Species-specific protection, site protection and/or management plans are urgently needed.

## 4 Conclusion

### 4.1 Habitat Conservation

The habitats of the relict species are mostly covered by Annex I habitat types. This means that even if a species itself is not directly protected, there is an indirect habitat protection in the Natura 2000 network of protected areas. Relict species are sensitive to climate change because they are generally strongly adapted to special conditions. Because of an increase in the anthropogenic causes of climate change, there is a high risk of extinction particularly for glacial relict species, as these represent the largest group of relict species in Germany. The best way to protect these local relict species is to include them in special management conservation plans. For many Natura 2000 sites, management plans should be set up and the relict species in Natura 2000 sites should be integrated as typical species where they occur.

Altogether, relict species from the analysed groups can be found in 46 habitat types of the Directive. At least 60–80% of the total area of most of these habitat types has been included in Natura 2000 sites. The most important habitat types hosting these relict species are bogs and species-rich grasslands, mainly of mountain ranges. In addition, screes represent an important glacial refuge for other relict species groups such as spiders. The importance of bogs has been pointed out in this

study for several taxa, but screes were only detected to be important for higher plants among the studied groups. In the latter, the most important habitat types referring to the studied groups were the following:

- 6210 semi-natural dry grasslands and scrubland facies on calcareous substrates (Festuco-Brometalia)
- \*7110 active raised bogs
- 7120 degraded raised bogs still capable of natural regeneration
- 7140 transition mires and quaking bogs
- 7230 alkaline fens
- 8210 calcareous rocky slopes with chasmophytic vegetation.

## 4.2 Species Conservation

One hundred and fifty-seven relict species in the taxonomic groups of higher plants, molluscs, dragonflies, damselflies and butterflies have been analysed in this study. Of these relict species, 143 are not covered by the direct species protection regime or have to be formally integrated into designated sites (not listed in Annexes II and IV of the Habitats Directive). A total of 14 species can be found in Annexes II, IV and V of the Habitats Directive (Table 7). Two of these species are extinct in Germany (*Saxifraga hirculus* and *Coenagrion hylas*), nine species are included in Annex II. The largest group of relict species in Germany directly listed in the Annexes of the Habitats Directive is molluscs (five species). Most relict species have no contact with other populations. Thus, they are isolated without gene flow between the remaining populations, so that they often show criteria that qualifies them as candidates for ESUs; however, more research work is needed on this topic. In accordance with the concept of assessing the responsibility of a region for the worldwide conservation of species (Gruttke et al. 2004), relict species are identified as conservation items of high priority.

Relict species in Germany can mostly be protected by the network of Natura 2000 sites as characteristic or typical species for habitat types of Annex I rather than by direct species protection from Annexes IV and II. Only nine species are listed in Annex II, so SCIs are being established or provided for seven of these nine species, because two species are extinct in the wild in Germany. The refuges of

**Table 7** Relict species in four taxonomic groups in Germany

	Number of species in Germany	Relict species	Relict species listed in the annexes of the Habitats Directive
Plants (without mosses and lichens)	ca. 3,500	100	4
Molluscs	342	38	5
Dragonflies and damselflies	81	7	2
Butterflies (Rhopalocera)	188	12	3

most relict species in Germany are at present protected by coverage through the network of Natura 2000 sites conserving the habitats of Annex I, in which the highest number of relict species occur. Because of climate change and their often fragmented and very small survival areas, relict species are subject to a high risk of extinction; so management plans should be provided for their conservation. For a few species, additional sites would be desirable to adequately cover their habitats within Natura 2000. Because the influence of anthropogenic climate change is increasing rapidly, management plans for the protection of relict species should be provided. The urgent need for management plans or species action plans is evident when considering the results of the current conservation status (German National Report, Nationaler Bericht 2007) of relict species listed in the Annexes of the Habitat Directive: two species are extinct and nine of the 14 species are identified as having an unfavourable - inadequate or unfavourable - bad conservation status in Germany (Tables 2–6).

**Acknowledgement** We would like to thank the LfU Bavaria for providing the distribution data for *Lycaena helle* in Bavaria and Ms. Ersfeld (BfN) for her great support during the production of the maps. Jan Habel (Trier), Petra Dieker (Trier), Aline Finger (Eglisau, Switzerland), Klaus Fischer (Greifswald) and Wolfgang Wipking (Cologne) gave us information about the recent distribution of *Lycaena helle* in Germany. We thank Nora Wuttke, Bonn, for support in English. Finally, we would like to thank one unknown reviewer for helpful comments.

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# Ecological limits *vis-à-vis* Changing Climate: Relic *Erebia* Butterflies in Insular Sudeten Mountains

Martin Konvička, Jiri Benes, and Thomas Schmitt

**Abstract** Insular middle mountains of Central Europe, such as Jeseník Mts. (max. altitude 1,495 m) and Krkonose Mts. (1,602 m), both in the Sudeten system, are poor in relic species, but the relic populations are suitable for studying the population responses to recent climatic change. There are two relic butterflies that are native to Jeseník Mts., each traditionally classified as an endemic subspecies: *Erebia sudetica sudetica* (Staudinger, 1851), a species protected by the EU Habitat Directive, and *E. epiphron silesiana* Meyer & Dür, 1852. Whereas Jeseník *E. epiphron* forms continuous populations, restricted to summit *Nardus*-dominated grasslands and not entering forested elevations, the distribution of Jeseník *E. sudetica* forms an archipelago at wet timberline sites and at clearings within mountain forests. Although both species avoid mountain forests, their lower distribution limits seem to be mediated by a limited range of available habitats, rather than of direct ecophysiological constraints. A non-native population of *E. epiphron* in Krkonose, transferred from the Jeseník in the 1930s, descends to ca 1,100 m, colonizing large clearings with cultural hay meadows within the forest belt. There is also behavioral and molecular evidence that *E. epiphron* cannot disperse through continuous forest. On the other hand, *E. sudetica* has a better dispersal power, and recent monitoring of its native Jeseník distribution suggests active colonization of woodland clearings. Thus, both butterflies can survive an altitudinal increase of the timberline, provided open habitats within the forests are secured. This conjecture suggests that these two and other alpine species survived past warmer periods of the Holocene in mountains, such as Jeseník owing to disturbance dynamics that never allowed complete forest closure. In the higher Krkonose, in contrast, they probably lacked suitable open habitats because of the encroachment of such sites by *Pinus mugo* dwarf pine, which is native to Krkonose but non-native to Jeseník.

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## 1 Introduction: Why Study Relics in Middle High Mountains

Isolated mountains of the temperate zone that overtop the timberline in their highest altitudes offer several advantages for studying the biology of alpine relics. In Europe, the subalpine summit habitats of the “middle high mountains” (Jenik 1998) include the uplands of Northern England and Scotland, the French Massive Central, and the highest parts of the Hercynian system in France (Vosges), Germany (Harz, Schwarzwald, Bayerischer Wald), and the Sudeten Mountains at the Czech/Polish border region (Krkonoše Mts. in the West, Kralický Sněžník and Jeseník Mts. in the East). Due to their small extents of (sub)alpine habitats, studies on alpine relics occurring in such systems can address questions that would not be tractable in the much larger and considerably higher systems of the Alps, Carpathians, or Pyrenees. In particular, the low richness of alpine elements in these small mountain areas reduces the complexity of biotic interactions, their small total areas facilitate research, and the geographic isolation from other mountain systems ensures that local populations do not interact with conspecific populations elsewhere. Relic species inhabiting middle high mountains are particularly vulnerable to habitat loss, aggravated by the ongoing climate change (Hill et al. 2002; Franco et al. 2006).

High mountain insects are subject to ecophysiological constraints, confining their distributions to high altitudes (Hodkinson 2005). Assuming that the outcome of warming climate results in a vertical shift in vegetation belts (e.g., Kullman 2006; Jurasinski and Kreyling 2007), alpine relics occurring above the timberline can be threatened in two ways, either directly by a shift of climatic conditions beyond their physiological limits, or indirectly, via habitat loss (e.g., Grabherr 2003; Holten 2003; Virtanen 2003; Virtanen et al. 2003). Either way, they should respond by uphill shifts and tracking of suitable climate conditions (e.g., Hill et al. 2002; Konvička et al. 2003; Wilson et al. 2007) and these displacements will eventually hit the upper limits in mountains not high enough to accommodate all the species occurring there now. Still, there had been other periods of relatively warm climate during the Holocene, especially the Atlanticum when the temperature was 1–2 degrees warmer than at present (Prentice et al. 2000), and there is little doubt that all the Northern and alpine relics occurring in European middle high mountains survived these warmer periods in situ, within the mountains where they occur today.

Two relic butterflies inhabit the highest altitudes of the Sudeten mountains: *Erebia epiphron* (Knoch, 1783) and *E. sudetica* Staudinger, 1851 (both Nymphalidae: Satyrinae). Taxonomists treat these populations as an endemic subspecies, *E. epiphron silesiana* (Meyer et Dür, 1852) and *E. s. sudetica* Staudinger, 1851, both belonging to species with considerably wider distributions elsewhere. Therefore, the Sudeten subspecies display extremely restricted native ranges: Exclusively the Jeseník Mts. in the case of *E. epiphron* and Jeseník Mts., Rychlebské Mts. and Kralický Sněžník Mts. in the case of *E. sudetica* (Kuras et al. 2001c). An artificially established population of *E. epiphron* occurs in the Krkonoše Mts., Western Sudetens. The closest native populations of the two species occur 200 km apart in the Carpathian High Tatras,

Slovakia (*E. epiphron*) and 600 and 800 km apart in the Rodna Mts., Eastern Carpathians, Romania and Grindelwald, Berner Oberland, Switzerland, respectively (*E. sudetica*).

Butterflies with such a restricted distribution have long attracted the attention of lepidopterists (e.g., Schwarz 1948; Stiova 1988). A systematic investigation of their ecology, however, began as late as the mid-1990s, when T. Kuras and J. Benes, then from Palacky University, Olomouc, initiated their mark-recapture surveys. This gradually extended to studies of behavior and population genetics, and to collecting additional information on the ecology of *E. euryale* (Esper, 1805) and *E. ligea* (Linnaeus, 1758), two *Erebia* species inhabiting the Sudeten mountain forests. Inclusion of *E. sudetica* to the EU Habitat Directive recently inspired annual monitoring of its sites. At present, Sudeten *Erebia* is among the most thoroughly studied butterflies in Central Europe.

Here, we review the ecological information available to date, focusing on the prospects of populations *vis a vis* recent climate change. We document that the population structure of *E. epiphron* and *E. sudetica* depends on the spatial distribution of their habitats. We then describe the outcomes of a notable experimental release of *E. epiphron* to the previously unoccupied Krkonose Mts., conducted in the 1930s. Recent surveys of the Krkonose population revealed that under certain conditions, this alpine butterfly can colonize altitudes below its subalpine habitats, and the same applies for *E. sudetica*, which occurs in large numbers at damp openings within the forest belt of Jeseník Mts. These observations suggest that although there exist ecophysiological limits to down-slope distribution of both species, their current distribution within the Sudeten system depends more on habitat availability than on climate.

## 2 The Biogeography of the Four Mountain *Erebia* Species of the Sudeten Mts

Of the four *Erebia* species occurring in the Sudeten Mts., *E. epiphron* is the most typical alpine species, inhabiting all the large high mountain systems of Europe, and also the Cantabrian range, the Massif Central, the Vosges, the Harz (extinct), mountains of the Western Balkans, and the Jeseník Mts. (Varga and Schmitt 2008). Allozyme studies unravelled the existence of at least five strongly differentiated genetic lineages in Europe: one confined to the Eastern Pyrenees, one in the central Pyrenees and Western Alps, two along the Southern and Southeastern Alps, and one in the Northern Alps and the Jeseník Mts. (Schmitt et al. 2006). The Carpathian populations are morphologically well differentiated from all other populations (Varga 2001). These molecular and morphological characteristics support that *E. epiphron* had several glacial retreats at the foothills of some mountain systems and others between mountain systems, as between the Pyrenees and Western Alps and between the Northern Alps and the Jeseník Mts., thus explaining the strong geographic disjunctions among some of the lineages of the species (Schmitt et al. 2006).

In contrast, *E. sudetica* is missing from most parts of the high mountain systems and is only found in two limited areas of the Western Alps (department Isère, France and near Grindelwald, Switzerland) and in some very localized areas in the Romanian Carpathians, but the species occurs in the Massif Central and in the Eastern Sudeten Mts. (Kudrna 2002). Preliminary allozyme analyses (Haubrich and Schmitt 2007, unpublished data of the authors) show that the populations from the Swiss Alps and the Sudeten Mts. are differentiated; however, the relation between the latter and the Romanian populations is still unknown.

*E. euryale* is a forest or forest skirt species (Sonderegger 2005) distributed all over the European mountain forest biome (Kudrna 2002). An allozyme study showed that this species is differentiated in at least four well supported genetic lineages: Pyrenees, Western Alps, Eastern Alps, and Southeastern Europe, and thus traces the glacial refugia of the mountain forest biome in Europe (Schmitt and Haubrich 2008).

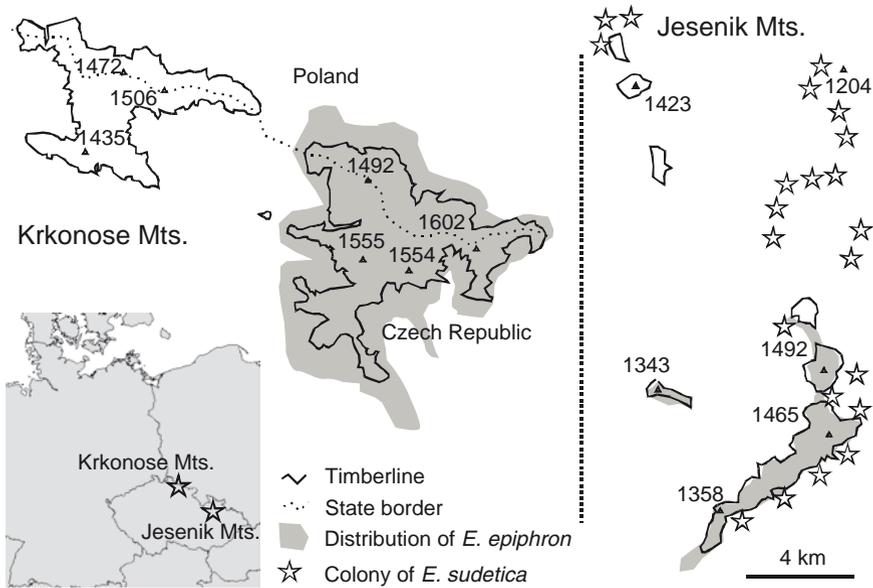
The boreo-montane *E. ligea* is distributed all over the mountainous areas of central and Eastern Europe, and in the lowlands of Northern Europe (Kudrna 2002). No genetic data over major parts of the distribution of the species are available. However, the North-South disjunction in this species is most probably a postglacial phenomenon (Varga and Schmitt 2008).

### 3 Jeseník Mts.: Geometry of Habitats Affects Population Structures

With timberline (formed by sparse growths of Norwegian spruce) at about 1,250 m a.s.l. and with the highest summit (Praded Mt., at 1,495 m), Jeseník Mts. do not contain alpine habitats *sensu stricto*. The flat plateaux above the timberline harbors 12 km<sup>2</sup> of subalpine grasslands, dominated by *Nardus stricta*, *Calamagrostis villosa*, and *Festuca supina*. These Nardeta of the Jeseník plateau represent essentially stabilized habitats not affected by succession (German: Dauergesellschaften). Steeper, wind-shielded valley headwalls affected by periodic avalanches harbor structurally diverse and species-rich tall-herb formations (Jeník 1961; Jeník et al. 1980).

The relic *Erebia* species of this mountain area differ in habitat use. *E. epiphron* occurs in high abundance at these stable subalpine grasslands, but rarely descends to elevations near the timberline, whereas *E. sudetica* is limited to the islets of timberline-adjointing tall-herb formations, which are transitional habitats in a gradient of natural succession (Fig. 1). The two remaining *Erebia* species (*E. euryale* and *E. ligea*) inhabit openings within mountain forests, ranging from ca 700 m to growths of dwarfed spruce at about 1,300 m.

Although these patterns were already noted by Stiova (1988), the first quantitative support originated from a trapping study (Benes et al. 2000). This study used flower-mimicking water-filled traps, an efficient method for capturing Lepidoptera of subalpine habitats. A total of 35 traps exposed for 665 trap-days captured 3,948



**Fig. 1** Map showing the position of Jeseník and Krkonoše Mts. in Central Europe (lower left corner), extend of subalpine habitats, and the distribution of two alpine butterflies, *Erebia epiphron* and *E. sudetica*. For *E. epiphron*, the entire distribution extend is shown, whereas for *E. sudetica*, the map shows only the larger colonies in Jeseník Mts. The northernmost Jeseník colonies shown are those in Ramzova saddle, which separates Jeseník (South) and Rychlebské Mts. (North)

butterflies and moths, including 3,731 *Erebia* individuals. The tall-herb formations near the timberline yielded the highest species diversity, corroborating patterns known for vegetation (cf. Jeník 1961, 1998). There was a significant habitat separation among woodland-preferring *E. ligea* and *E. euryale*, timberline-preferring *E. sudetica*, and grassland-preferring *E. epiphron* (Kuras et al. 2000).

The summit grasslands form several islets in Jeseník. *E. epiphron* inhabits the two largest units, main ridge (area 10 km<sup>2</sup>) and Mravenecník (area 1.2 km<sup>2</sup>), both separated by 4 km of forested saddles (Fig. 1). In contrast, the tall-herb formations inhabited by *E. sudetica* form an archipelago of valley headwalls and springs. Kuras et al. (2003) used the mark-recapture method to illustrate that the differences in spatial configuration of habitats determine the population structures of both species. The main ridge population of *E. epiphron*, studied by mark-recapture in 1996, was really huge: For a total of 4,034 marked individuals, only 236 individuals were recaptured. This was likely due to rather high local density, as the estimated numbers were about 20,000 males for 1 km<sup>2</sup> of habitat, corresponding to hundreds of thousands of individuals for the entire main ridge (female numbers were not estimable), and the unsuitable mountain weather conditions not permitting daily field work. For the lateral Mravenecník population, marking for 8 days in 1997 yielded 2,542 captured and 440 recaptured individuals, giving an estimate of 4,500 individuals.

In the case of *E. sudetica*, one large and one small population were studied in 1998, the large one yielding 2,171 captured and 893 recaptured individuals, the small one yielding 253 captured and 80 recaptured individuals. The estimates were 5,000 and 300 individuals, respectively, suggesting a population size for the entire main ridge area of around 10,000 individuals.

*E. epiphron* individuals were considerably poorer dispersers than those of *E. sudetica*. The lifetime probability of moving 1 km was 0.5–1.2% for the former and 2.2–6.4% for the latter, the wide range being due to differences among sexes and populations (Kuras et al. 2003). The difference became even more dramatic over longer distances: the probability of crossing 5 km was  $1 \times 10^{-10}$  for *E. epiphron* and  $1 \times 10^{-7}$ , thousand times higher, for *E. sudetica*. The latter species is thus more mobile, indicating that populations inhabiting relatively unstable environments, such as disturbance-dependent timberline sites, should profit from occasional migrations, while those inhabiting stable and extended environments, such as the summit grasslands, may do without them.

#### 4 Krkonose *E. epiphron*: Novel Biotopes and Dispersal Limitations

Compared to Jeseník, Krkonose Mts. are higher (max. altitude: Snezka Mt., 1602), contain larger subalpine areas (about 50 km<sup>2</sup>) and rocky valleys resembling those of much higher mountains. The main difference in vegetation is a native occurrence of *Pinus mugo*, a dwarf pine forming a distinct belt sandwiched between spruce forests and subalpine grasslands.

Krkonose lacked truly alpine *Erebia* until the early 1930s, when the entomologist Josef Soffner sent two parcels, each containing 50 *E. epiphron* and 50 *E. sudetica* females, from a Jeseník site to two Krkonose inn-keepers, asking them to release the butterflies in the wild. The target inns were Lucni bouda (1,410 m.) amidst summit grasslands and Rychorska bouda (1,000 m), a rather uncharacteristic site of pastures below the timberline.

Not much was heard about the releases until the 1960s, when Borkowski (1966) reported *E. epiphron* from Krkonose Northern slopes, about 1 km from the Lucni bouda release site. Soffner (1967) responded by publishing a paper describing the circumstances of the 1930's transfer. Gradually, the butterfly became very abundant within its new range (Liska and Skyva 1997; Cizek et al. 2004). In contrast, neither the released *E. sudetica* nor the other release of *E. epiphron* established a population. It is evident from hindsight that Soffner did not understand the precise habitat preferences of the two species, and selected inappropriate settings for both *E. sudetica* releases and for the Rychorska bouda release of *E. epiphron*.

For decades, there was no systematic interest in the established *E. epiphron* population, despite some fascinating questions brought about by its existence. Specifically, the bottleneck during the transfer could have impaired the adaptive ability of butterflies. On the other hand, the release area contains a broader range

of biotopes than the native Jeseník area, which might allow expansion to novel habitats. Last but not least, tracking the newly established population could have allowed discerning possible dispersal barriers.

Cizek et al. (2003) approached these questions in a trapping study, similar to the earlier Jeseník study by Benes et al. (2000). Series of traps were set to all main Krkonose biotopes, from clearings deep in the forest belt up to the rocky summits. The mountains were also systematically surveyed to ascertain the current distribution extent. As in Jeseník, *E. epiphron* abundances peaked at subalpine grasslands near 1,300 m. The species nevertheless reached the uppermost elevations at about 1,600 m and, more surprisingly, has colonized open clearings and hay meadows below the timberline, down to about 1,100 m. Compared to the Jeseník population, the Krkonose population in fact expanded both uphill and downhill.

An important finding concerned the limits of the expansion. Soffner released the butterfly at the Eastern ridge of the Krkonose Mts., which is completely colonized now. The Western ridge, similar to the Eastern ridge in the area of subalpine habitats and maximum elevations, remained uncolonized for over 75 years. The two ridges are separated by about 4 km (aerial distance) of forested elevations, which apparently acted as a dispersal barrier. The butterfly failed to cross the forests – while colonizing the non-forested sites below the timberline.

Another evidence for avoidance of forests originated from a study of the behavior of *E. epiphron* and *E. euryale*, conducted in sympatry at the Jeseník main ridge (Konvička et al. 2002). We recorded the behavior along repeated transects, together with time of day, weather, and vegetation where activities took place. Whereas *E. euryale* utilized woody structures, such as dwarfed spruce, for activities as resting and basking, *E. epiphron* restricted all its activities to open grasslands.

Further evidence that forests restrict *E. epiphron* dispersal originated from a genetic study of the native and introduced populations (Schmitt et al. 2005). The two native populations within Jeseník were significantly differentiated, as were the source population in Jeseník and the introduced population in Krkonose. The genetic distance between the Jeseník source and the Krkonose target sites was less than the distance between samples from northernmost and Southernmost limits of Jeseník main ridge. This revealed isolation by distance along the ridge, consistent with rather limited dispersal. The smaller lateral Jeseník population was strongly genetically impoverished. Its mean heterozygosity was half of the mean heterozygosity of three samples taken along the main ridge (5.5 vs. 10.0%), and similar patterns applied to the total percentages of polymorphic loci, polymorphic loci with the most common allele not exceeding 95%, and the mean number of alleles per locus. No such impoverishment applied to the introduced Krkonose population, indicating that the 50 females transferred by Soffner sufficed to transfer the entire (allozyme) allelic variation of the Jeseník source population.

Perhaps the most intriguing result of the population transfer was the colonization of lower-elevation habitats in Krkonose, as opposed to Jeseník (Cizek et al. 2004). This is explicable by different levels of fragmentation of mountain forests in the two mountains, resulting in different availability of non-wooded habitats within their forested elevations. A peculiar feature of the Krkonose landscape is “cultural”

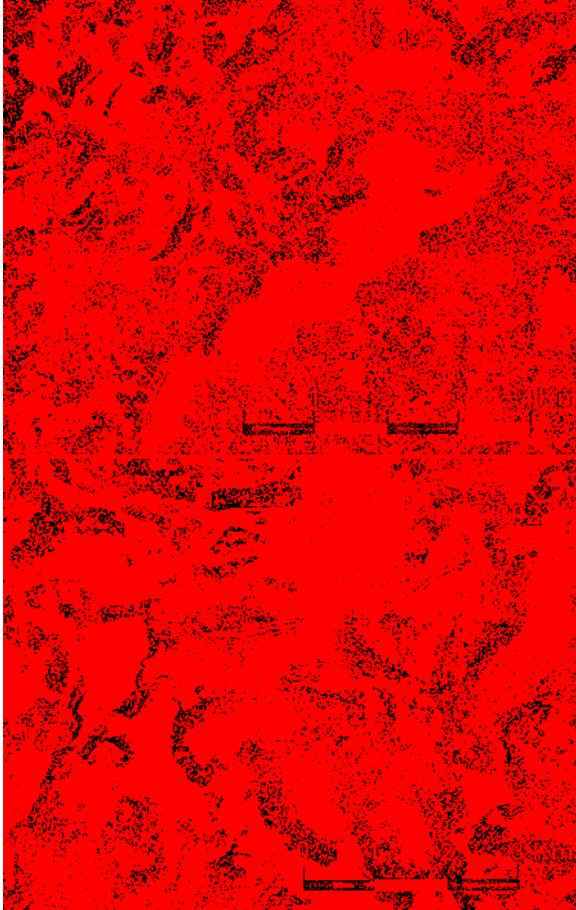
meadows and pastures, created by forest clearance in altitudes of 800–1,200 m (Krahulec et al. 1997). Moreover, much of Krkonose mountain woodlands fell victim to “salvation logging” of the 1980s, which reacted to die-backs caused by atmospheric pollution (cf. Emmer et al. 1998). The forests are thus dissected by a dense network of open habitats. These are practically missing in the forested elevations of Jeseník, making Jeseník mountain forests more restrictive for dispersing *E. epiphron* than Krkonose forests (Fig. 2).

The colonization of elevations near 1,100 m in Krkonose, but not in Jeseník, documents that a lower climatic elevation limit of *E. epiphron* is situated in altitudes suitable for tree growth. The current lower distribution limits are thus not determined solely climatically, but also by the availability of open grasslands below the timberline.

## 5 Jeseník *E. sudetica*: Disturbance-Dependent Metapopulation

The known distribution of Sudeten *E. sudetica* extends beyond the timberline sites in Jeseník Mts., and the species also inhabits much lower Rychlebské Mts. (highest altitude Smrk, 1,125 m) (cf. Kuras et al. 2001a,c, Maslowski 2005). Based on the then-available information, Kuras et al. (2003) speculated that the populations of the more ephemeral habitats below the timberline are only transitory, depending on colonists originating from the more stable timberline sites colonizing the temporary woodland openings.

Recently (2007), we surveyed as many as possible forest-belt localities of *E. sudetica* in order to ascertain their status and prospects. We spent over 30 person-days visiting such habitats as clearings, open tracks, and meadows along valley streams. Additional surveys covered Rychlebské and Kralický Sněžník Mts. We failed to confirm the species for the latter two mountains, but we found dozens of extant colonies within the Jeseník forest belt, the lowest-situated ones at 700 m a.s.l. They included boggy hay meadows in saddles and along stream sides, woodland clearings opened up during salvage logging operations in the 1980s and 1990s, and linear open structures such as those below ski lifts. All sites displayed high humidity, typically provided by groundwater springs. Colonies near lower distribution limits, most of them on meadows, tended to be small, hosting just a few individuals. In contrast, colonies in altitudes near 1,000 m, most of them on muddy clearings, were highly abundant. At several sites, we counted several hundreds of individuals per 100 m of transect walk, corresponding to thousands of individuals present. These very large colonies apparently profited from the forest decays in the 1980s and subsequent salvage logging. The conditions were remarkably similar to the clearcuts in Krkonose, with the difference that the Krkonose clearcuts hosted *E. epiphron* that reached them from adjoining subalpine grasslands. Probably because all the Jeseník clearcuts were separated by forested areas from the subalpine sites, they exclusively hosted *E. sudetica*, again demonstrating the good dispersal ability of *E. sudetica* and the poor dispersal ability of *E. epiphron*.



**Fig. 2** Aerial photographs of Jeseník main ridge (*above*) and a section of Krkonose Eastern ridge (*below*), with white line showing the position of timberline. Well apparent are the starkly different levels of fragmentation of forest zones of the two mountains

The existence of large forest belt colonies of *E. sudetica* has changed our view of relative conservation importance of these low-elevation sites. It is now clear that the forest belt colonies are scattered over an area of about 200 km<sup>2</sup> and that the total number of butterflies inhabiting these colonies exceeds the number inhabiting the timberline sites. The dynamics of these colonies, however, remains unknown. The forest-belt populations probably form a dynamic metapopulation system, tracking occasional forest openings and forming transient colonies there (see Bergman 2001; Wahlberg et al. 2002, for other examples). The relative role of the timberline vs. forest-elevation sites as source of the colonists remains unknown: Does the entire system depend on the relatively permanent sites near the timberline, or is it essentially self-sustaining, depending on transfers of colonists among forest-elevation patches? Do some “source” sites exist within the forested elevations as well, perhaps

at streamside bogs in mountain saddles? Some of the candidate permanent sites could have been heavily altered during the last decades: for example, a pass (800 m) separating Jeseník and Rychlebské Mts. still hosts some colonies at boggy meadows, but the extent of the meadows shrank to tiny remnants during the last few decades as a result of building development, afforestation, and grassland drainage.

However, the ability to occur on open sites below the timberline testifies that similar to *E. epiphron*, *E. sudetica*'s lower limit is habitat-limited rather than climate-limited. Its lowest-elevation populations are small, and we cannot distinguish at this moment whether this is due to the occurrence near a lower climatic limit, or due to the restricted habitat availability. However, in elevations from about 900 m onwards, all clearings and boggy meadows tend to be occupied, usually by high densities of butterfly.

## 6 Warming Climate, *P. mugo* and Future of Sudeten *Erebia*

The long-term persistence of these two Sudeten species of *Erebia* butterflies will depend on the continuous presence of suitable open habitats in the upper parts of the mountains. The current distribution patterns suggest that the habitats do not necessarily need to be located above the climatic timberline. Also, none of the species is threatened by direct destruction of habitats, as most of their sites already enjoy legal protection, either in the form of reserves within the Jeseník-protected landscape area, or as a core zone of the Krkonose/Karkonosze national parks. The populations are abundant and/or interconnected, further adding to the rather optimistic prospects.

As revealed by pollen and macrofossil records, trees and shrubs ascended to higher elevations, possibly by as much as 100 m, at the height of the Atlanticum period (Jankovská 2004; Tremel et al. 2008). This likely diminished the extent of subalpine habitats, relative to the current situation. However, because the specialized alpine biota, including the *Erebia* butterflies in Jeseník, had survived the period in situ, the summit habitats could not be closed-canopy forest, but rather a patchwork of tree growths and open sites. In the same vein, the current diversity of alpine relics might survive the recent warming episode, provided the warming does not get too extreme and the habitats in the uppermost elevations remain rather open. The processes that control tree growth must be adopted by conservationists in order to support the threatened relic species.

An important aspect concerns the species richness in alpine relics in Krkonose and Jeseník. Despite containing much smaller subalpine area, Jeseník hosts approximately as many relic Lepidoptera as do Krkonose (Table 1). The Krkonose fauna is relatively poor, apparently due to the presence of the species-poor vegetation belt of *P. mugo*. The dwarf pine used to cover even larger areas than in present before it was cleared by humans to obtain pastures, and there is no doubt that it expanded to still higher altitudes during the climatic optimum. In this period, relic alpine biota likely retracted to rocky slopes and scree, unsuitable for pine growth. Interestingly,

the pine is not native to Jeseník, but had been planted there at the turn of the 20th century. Thus, the existence of *P. mugo* might explain the lack of some relic Lepidoptera associated with grassland formations in the Krkonose Mts., such as both species of *Erebia* and two *Clepsis* tortricids. On the other hand, several species absent from Jeseník are associated with the rocky habitats (*Elophos operaria*,

**Table 1** List of all Lepidoptera species associated with high mountain (subalpine and alpine) habitats and recorded from Krkonose (KRK) and Jeseník (JES) Mts., Czech Republic

Species	Family	KRK	JES	Distribution
<i>Incurvaria vetulella</i> (Zetterstedt, 1839)	Incurvariidae	+	+	A, B, C
<i>Lampronia rupella</i> (Dennis et Schiffermüller, 1775)	Prodoxidae	+	+	A, B, C
<i>Argyresthia amiantella</i> (Zeller, 1847)	Yponomeutidae	+	–	A, C
<i>Rhigognostis senilella</i> (Zetterstedt, 1839)	Plutellidae	+	–	A, B, C
<i>Denisia nubilosella</i> (Herrich-Schäffer, 1856)	Oecophoridae	+	+	A, C
<i>Elachista kilmunella</i> Stainton, 1849	Elachistidae	+	+	A, B, C
<i>Chinodes viduella</i> (Fabricius, 1794)	Gelechiidae	+	+	A, B
<i>Sparganothis rubicundana</i> (Herrich-Schäffer, 1856)	Tortricidae	–	+	B
<i>Clepsis steineriana</i> (Hübner, 1799)	Tortricidae	–	+	A
<i>Clepsis rogana</i> (Gueneé, 1845)	Tortricidae	–	+	A, B, C
<i>Phiaris obsoletana</i> (Zetterstedt, 1839)	Tortricidae	+	–	A, B, C
<i>Pseudococcyx mughiana</i> (Zeller, 1839)	Tortricidae	+	–	A, C
<i>Eudonia petrophila</i> (Standfuss, 1848)	Crambidae	+	+	A, C
<i>Eudonia sudetica</i> (Zeller, 1839)	Crambidae	+	+	A, B, C
<i>Catoptria maculalis</i> (Zetterstedt, 1839)	Crambidae	+	–	A, B, C
<i>Catoptria petrificella</i> (Hübner, 1796)	Crambidae	–	+	A, C
<i>Udea alpinalis</i> (Dennis et Schiffermüller, 1775)	Crambidae	+	+	A, C
<i>Erebia epiphron</i> (Knoch, 1783)	Nymphalidae	– <sup>a</sup>	+	A, C
<i>Erebia sudetica</i> Staudinger, 1861	Nymphalidae	–	+	A, C
<i>Isturgia roraria</i> (Fabricius, 1776)	Geometridae	–	+	A, C
<i>Elophos operaria</i> (Hübner, 1813)	Geometridae	+	–	A, C
<i>Psodos quadrifaria</i> (Sulzer, 1776)	Geometridae	+	–	A, C
<i>Glacies alpinata</i> (Scopoli, 1763)	Geometridae	+	+	A, C
<i>Perizoma obsoletata</i> (Herrich-Schäffer, 1838)	Geometridae	+	–	A, C
<i>Eupithecia silenata</i> Assmann, 1848	Geometridae	+	+	A, C
<i>Apamea maillardi</i> (Geyer, 1834)	Noctuidae	+ <sup>b</sup>	–	A, B, C
<i>Xestia alpicola</i> (Zetterstedt, 1839)	Noctuidae	+	–	A, B, C
<i>Diarsia mendica borealis</i> Zetterstedt 1839 <sup>c</sup>	Noctuidae	–	+	B
Total		20	18	

Based on Liska (2000), with nomenclature modified after Karsholt and van Nieuwerkerken (2004). “Distribution” describes extra-Sudeten distribution of individual species, in which A=occurrence in the Alps and other mountains of W and C Europe; C=occurrence in the Carpathians and other systems of SE Europe; B=occurrence in Northern Europe

<sup>a</sup>Intentionally introduced population: see text for details

<sup>b</sup>A single record, doubted by some authors

<sup>c</sup>A population with diurnal activity, likely distinct from “lowland” *D. mendica*. Its exact status needs clarification

*Catoptria maculalis*), a habitat mostly absent from the Jeseník Mts., but existing in the Krkonose Mts.

Another important aspect concerns the grasslands. Recently, there is much effort to restore the “cultural” grasslands in the Sudeten Mts. by reinstalling the traditional management, and the prospects are optimistic (e.g., Krahulec et al. 1997; Pavlu et al. 2003; Hejčman et al. 2005, 2006). However, the natural subalpine grasslands had also been managed for centuries by grazing and haymaking. Cessation of activities, and a resulting non-intervention regime, causes a gradual impoverishment of the vegetation, prevalence of a few dominant species, and accelerated trees and shrubs expansion (Klimeš and Klimešová 1991; Hejčman et al. 2006). Effects on *Erebia* species are not yet apparent, but may eventually manifest as direct loss of habitats. The non-intervention management is sometimes advocated as return to a “natural state,” which is a risky adventurism in the face of losses of sensitive species, and under the threat of warming-accelerated habitat changes.

*E. epiphron* requires long-term preservation of open grasslands, preferably interconnected to buffer for its poor dispersal ability. (Recall the decreased genetic variation of the isolated lateral Jeseník population). In Jeseník, where only the subalpine grasslands exist, blocking of tree growth, including the removal of non-native dwarf pine *P. mugo*, represents the only way to support its population. Because Krkonose are higher than Jeseník, the introduced population can be viewed as a genetic reserve, having a higher chance of survival should warming climate eradicate the species in Jeseník. Even here, ascending timberline should be kept in check, and cultural grasslands below the timberline should be managed using approaches mimicking the traditional meadow husbandry.

The situation of *E. sudetica* is more complex, but essentially similar. The structurally rich, “notched” timberline sites with sparse growths of trees may be ascending, but this will not necessarily threaten the populations unless the sites are artificially afforested by spruce or dwarf pine. Practically, all these timberline sites are fairly stable because they are periodically affected by avalanches, which keep them devoid of trees. The fate of the more ephemeral forest-belt populations seem less optimistic, as the vast clearings, now hosting the highest abundance of the species, will gradually succumb to forest re-growth. To support the forest-belt populations, natural disturbance factors opening up canopy, such as windfalls and landslides, should be welcomed rather than abhorred and battled against, and active steps to open the canopy structure at such sites as woodland spring areas and valleys of mountain brooks should be undertaken.

Any predictions on the future of the two butterflies are thwarted by the lack of understanding of ecophysiological limits of the species. So far, we understand that they need cool conditions for development, and it seems likely that the limits operate on requirements of winter larvae. We do not know, however, whether the required condition is sufficiently long allowing uninterrupted winter freezing for uninterrupted hibernation, or whether sufficient height and duration of isolating snow cover is necessary. In the first case, the larvae would be threatened by osmotic stress if the winter gets warmer, while in the latter, they may die from frosts if the snow covers diminish (cf. Hodkinson 2005). The mechanisms of cold (or heat)

tolerance of both species are tractable by experimentation and the findings will be critical for forecasting the responses of both species to changing climate.

**Acknowledgement** We thank O. Cizek, T. Kuras and P. Vrba for inspiring discussions. Field studies of Sudeten *Erebia* were supported by Palacky University Olomouc, and by Jeseníky Landscape Protected Area and Krkonoše National Park administrations; particular thanks to conservation officers P. Balaz, V. Kavalcova, and J. Vanek. Molecular study of *E. epiphron* was supported by Grant Agency of the Czech Republic (B6001306/2003). Monitoring of *E. sudetica* is supported by the Czech Conservation Authority, Grant Agency of the Czech Academy of Sciences (600070601) and Czech Ministry of Education (LC06073, 6007665801).

Due to their borderline position and the complex 20th century history, Sudeten Mts. have different names in different languages, which introduces much confusion to biogeographic literature. We present below a simple translation guide for the toponyms appearing in the chapter. They are ordered alphabetically, according to valid names in Czech, and provided, if appropriate, with corresponding toponyms in German [D], English [GB], and Polish [PL]. Jeseník (= Hrubý Jeseník, Jeseníky) [CZ]=Altwater Gebirge (=Hochgesenke) [D]; Kralický Sněžník [CZ]=Glatzer (= Grulich, Spiegglitzer, Großer) Schneeberg [D]=Śnieżnik Klodzki [PL]; Krkonoše [CZ]=Riesengebirge [D]=Giant Mts. [GB]=Karkonosze [PL]; Lucni bouda [CZ]=Wiesenbaude [D]; Praděd [CZ]=Altwater [D]; Rychlebské Hory [CZ]=Reichensteiner Gebirge [D]=Golden Mts. [GB] = Góry Złote [PL]; Rýchorská bouda [CZ]=Rehornbaude [D]; Sněžka [CZ]=Schneekoppe [D]=Śnieżka [PL]; Šumava [CZ]=Böhmerwald [D]

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# Conservation and Management of the Habitats of Two Relict Butterflies in the Belgian Ardenne: *Proclossiana eunomia* and *Lycaena helle*

Philippe Goffart, Nicolas Schtickzelle, and Camille Turlure

**Abstract** Habitat preservation and restoration are crucial for species conservation and to tackle its main challenges. In many cases, this implies management actions, especially for semi-natural ecosystems. We address here the question of management actions suitable for maintaining viable populations of two relict butterfly species inhabiting wet meadows and peat bogs in the Belgian Ardenne: the bog fritillary *Proclossiana eunomia* and the violet copper *Lycaena helle*. Habitat loss due to natural vegetation succession towards forest can be prevented either by mowing (traditional way of management) or by extensive grazing (with cattle or horses).

Results from field studies and experiments conducted on several sites in the Belgian Ardenne led to two conclusions. Mowing can strongly affect the populations of both species, the impact being particularly pronounced on larval stages due to the removal of grass tussocks and the export of hay containing host plant on which caterpillars feed. Grazing also has a negative effect on both species, with adult density being lower on grazed biotopes compared to abandoned ones. However, the negative impact is more limited for grazing than for mowing.

As both management methods have more or less pronounced negative impacts on populations of these two butterfly species, they should be applied with caution. We suggest that mowing should be applied in long-term rotation, with the preservation of sufficient refuge areas every year, and that grazing should be applied in late summer, every other year, with a low stocking rate (<0.2 LU/ha/year). More particularly, to ensure their effectiveness in maintaining a favorable state, both the biotope and the associated populations using it as their habitat, management actions must be designed according to the species habitat requirements, and adapted to carefully monitor their implementation.

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## 1 Introduction

In Europe, and particularly in Belgium, butterfly diversity has strongly decreased during recent decades (Van Swaay et al. 2006; Maes and Van Dyck 2001; Goffart et al. 1992; Fichet et al. *in press*); specialist species have been particularly affected. Of prime importance in this context is the conservation of remnant relict butterfly species. The wet meadows and peat bogs of the Belgian Ardenne high plateaus still offer ecological conditions close to those of Northern Europe, allowing populations of relict butterfly species to persist.

However, these populations, at the Southern margin of their distribution range, have a sparse and disjunct distribution (Kudrna 2002). Moreover, they are spatially trapped: they cannot shift their distribution range either to the North, because there is no suitable habitat patch between this region and other populations widespread in Northern Europe (Parmesan et al. 1999; White and Kerr 2006), or uphill because the upper altitudinal limit is reached in the region (Konvicka et al. 2003).

The bog fritillary *Proclossiana eunomia* and the violet copper *Lycaena helle* are two remarkable relict butterflies of the Ardenne massif. Their Belgian populations are amongst the most important of Western Europe and are genetically unique (Meyer 1980, 1981a,b; Barascud and Descimon 1992; Nève et al. 2000; Descimon et al. 2001; Finger et al. 2009). Moreover, they are in decline and threatened (Fichet et al. 2008), as is the case in other parts of Europe too, where *L. helle* is classified as “vulnerable” (van Swaay and Warren 1999) and recently added to the Appendices II and IV of the European Habitat Directive (92/43/CEE). Their preservation is, therefore, highly desirable.

All remnant populations of relict species in the Ardenne region inhabit pioneer more semi-natural biotopes. Without any disturbance, these biotopes are progressively invaded by shrubs and trees, turn to forest, and ultimately disappear. In extended ecosystems, natural disturbances, like fires or storms, can rejuvenate the biotope by converting it back to open vegetation (Bullock and Pakeman 1997; Svenning 2002). This is no longer the case anywhere in the Western Europe today because of the small area of such remnants. Therefore, human actions are clearly needed to mimic the effects of natural disturbances, through the use of management techniques such as mowing, grazing, and tree cutting (Sutherland 2002). Such management often positively affects the ecological processes that are crucial for biodiversity, by breaking the dominance of highly competitive plants, reducing bush encroachment, or making vegetation structure more varied (Ausden and Treweek 1995). Although these management methods are widely and more or less efficiently implemented on biotopes (WallisDeVries et al. 1998; Krebs et al. 1999), little is known about the detailed effects on butterfly populations and their resources.

In this context, our objectives were to assess the impact of two management techniques, mowing and grazing, on populations of *P. eunomia* and *L. helle*, which inhabit wet meadows and peat bogs in the Belgian Ardenne. Mowing was traditionally applied with non-mechanical means in the Ardenne alluvial meadows (Lambert 1963). Grazing was not used in Ardenne valleys, but is currently becoming popular among nature managers (Delescaille 2002), because of its ease of implementation.

We assessed for both species (1) the impact of mowing on larval stages (through the number of emerging adults) and on the attractiveness of mown biotopes for adults and (2) the impact of three distinct grazing regimes on the adult density. As resources and life history traits of the two species are different, we expected to find contrasted, perhaps even completely different, impacts of both management techniques according to species. Indeed, even if caterpillars of both species feed only on *P. bistorta* leaves, nectar use by adults is different: *P. eunomia* adults only feed on *P. bistorta* flowers, while *L. helle* adults use a wider range of nectar resources, from both herbaceous and woody species. Moreover, overwintering strategies are distinct (Barascud and Descimon 1992): *L. helle* spends the winter as a pupa, while *P. eunomia* spends the winter as a caterpillar. The related applied question is whether there is an efficient management regime for these two relict butterfly species at the same time.

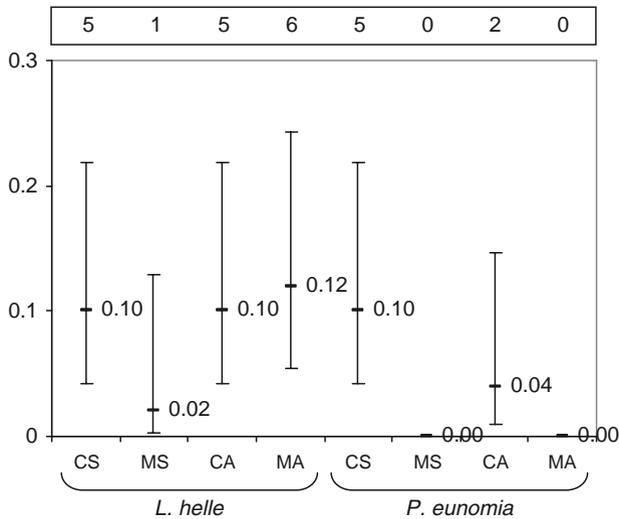
## 2 The Impact of Habitat Management Via Mowing

### 2.1 Experiments and Results

Two experiments were conducted in the “Prés du Sommerain” nature reserve (50°10'N 5°47'E) in the Belgian Ardenne to assess the impact of mowing: the first one on the larval stage and the second on the adult stage.

The first experiment aimed at assessing the survival of larval stages after summer and autumn mowing, through the number of emerging adults. Six pairs of 200 m<sup>2</sup> (25×8 m) strips with a dense cover of bistort (i.e., the host plant) were studied in an abandoned wet meadow between 1993 and 1995 (one pair in 1993, two in 1994, three in 1995). In each pair, one strip was mown (in summer between the end of June and the beginning of August, or in autumn in October) and the other strip was left untouched for control. One square meter emergence traps were set up above the vegetation at the end of April, the year after the mowing management. They were arranged in series of four traps, two traps being placed in the mown strip, the other two in the adjacent portion of the control strip. Over a period of three years, a total of 50 traps were devoted to each category (summer control, summer mowing, autumn control, autumn mowing). The presence of emerged butterflies in the trap was then checked daily until the end of summer.

Logistic regression models (implemented using SAS software: [www.sas.com](http://www.sas.com)) showed no difference in the probability of emergence of adult butterflies between summer and autumn control strips for each species ( $\chi_1^2 = 1.43$ ,  $p=0.23$  for *P. eunomia*,  $\chi_1^2 = 0$ ,  $p=1$  for *L. helle*) (Fig. 1). Whatever the timing of mowing, summer or autumn, the probability of emergence was higher in the control strips than in the corresponding mown strips for *P. eunomia* (contrast “pooled control strips vs pooled mown strips”:  $\chi_1^2 = 8.62$ ,  $p=0.0033$ ; “summer control vs summer mown strips”:  $\chi_1^2 = 7.19$ ,  $p=0.0073$ ; “autumn control vs autumn mown strips”:  $\chi_1^2 = 2.81$ ,  $p=0.0935$ ); mowing had in fact a very drastic impact on *P. eunomia* as no adult ever



**Fig. 1** Impact of mowing treatment on the probability (with 95% confidence intervals) of adult emergence of the two butterfly species. CS summer control strips, MS summer mown strips, CA autumn control strips, MA autumn mown strips. Numbers of emerged individuals are presented in the inset

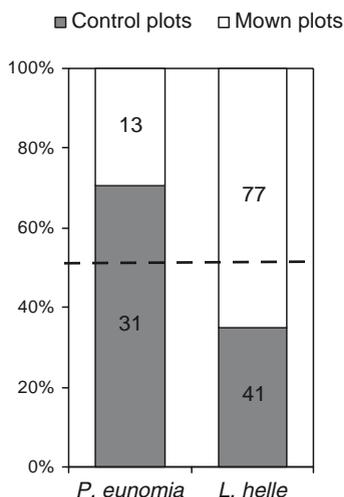
emerged in mown strips (Fig. 1). For *L. helle*, the impact of mowing on larval stages was different according to the timing of mowing (Fig. 1): autumn mowing showed no influence on the probability of emergence of adult butterflies ( $\chi^2_1 = 0.1$   $p=0.749$ ), but summer mowing seemed harmful ( $\chi^2_1 = 3.08$   $p=0.079$ ); the difference between summer and autumn mowing was significant ( $\chi^2_1 = 4.23$   $p=0.039$ ).

The second experiment aimed at comparing how mown and control plots attracted adult butterflies. Three plots of about 500 m<sup>2</sup> (25×20 m) were mown in summer 1994 and the same number of plots were left untouched for control. Several 15 min transect counts were performed during the flight period of both species in summer 1995. There was a difference in the relative attraction exerted by mown and control plots, significant for both species ( $\chi^2_1 = 7.36$ ,  $p=0.007$  for *P. eunomia*;  $\chi^2_1 = 10.98$ ,  $p<0.01$  for *L. helle*), but this response differed between species ( $\chi^2_1 = 16.55$ ,  $p<0.001$ ). *P. eunomia* adults seemed to avoid – or at least have a lower attraction towards – mown plots, while *L. helle* adults showed a significant attraction towards mown plots (Fig. 2).

## 2.2 Linking the Impact of Mowing with Habitat Requirements

The impact of mowing was clearly more pronounced on *P. eunomia* than on *L. helle*. The disastrous impact of mowing, both when done in summer and in autumn, on *P. eunomia* larvae is easily explained by the usual location of caterpillars,

**Fig. 2** Impact of mowing treatment on the attractiveness of the area for the two butterfly species. Shown are the numbers and associated percentages of adult butterflies of each species counted in control and mown plots



which find shelter preferentially in grass tussocks, from the beginning of summer until the next spring (Goffart et al. 2001). Indeed, grass tussocks appear to play a crucial role in caterpillar thermoregulation (Turlure unpublished data), and probably also allows them to escape from parasitoids, predators, and winter flooding (Joy and Pullin 1997; Severns et al. 2006). These tussocks are cut during mowing, and caterpillars very likely removed with the hay, causing direct mortality. Concerning the avoidance of mown areas – or at least the lower attraction towards the mown areas – displayed by *P. eunomia* adults, it may be related to the change of biotope physiognomy induced by mowing: grass-tussocks are cut and relief becomes almost flat, giving a less attractive vegetation facies for males and females (Turlure et al. 2009). This has been confirmed by another experiment, in which the same plots were manipulated by sequentially placing and removing grass tussocks during a single day, and comparing attractiveness for *P. eunomia* (Goffart unpublished data). Both sexes indeed actively search for tussocks: females find appropriate locations for egg-laying and males find a higher probability to encounter virgin (unmated) females at the precise location of their emergence.

For *L. helle*, the period of mowing proved to be crucial for the survival of larvae. The contrasted responses to summer and autumn mowing can be understood in the light of the butterfly life history: caterpillars, hanging under the leaves in summer, are likely to be carried away with the hay and suffer direct mortality, whereas the overwintering pupae, settling on the top soil in autumn, escape from later mowing. Survival of the pupae (and hence, emergence success) might be even higher in the mown areas, because the shorter vegetation height and associated change in micro-climatic conditions (Thomas 1991; Bourn and Thomas 2002) induced by mowing

could be more favorable for them. Furthermore, *L. helle* adults seemed to be attracted towards the mown areas. This was clearly linked to the higher density of some nectar flowers like *Cardamine pratensis* (Goffart et al. 2001), as the majority of adult butterflies were observed feeding on them. *L. helle* adults being attracted to mown areas where their offspring suffer from elevated mortality due to mowing actions, an “ecological trapping effect” (Shlaepfer et al. 2002; Battin 2004) might occur if mowing is applied every year in the stipulated period, i.e., in summer.

### 3 The Impact of Habitat Management Via Grazing

#### 3.1 Experiments and Results

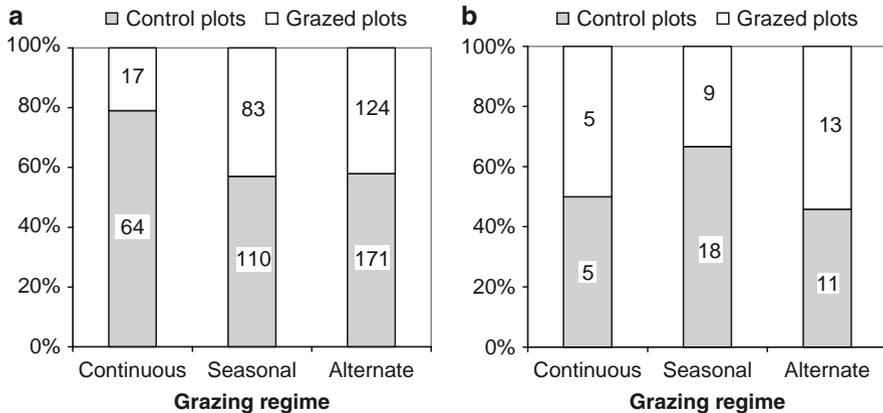
Experiments to assess the impact of light grazing were conducted on several sites in the Belgian Ardenne (Deifelt, Commanster, Shoenberg, Martelange, and Straimont). Within these sites, 14 plots larger than 2 ha were selected, eight lightly grazed (stocking rate <0.2 LU/ha/year) for at least 5 years and six abandoned (control). Three distinct forms of light grazing were distinguished: (1) continuous grazing, cattle being present all the year round; (2) seasonal grazing, cattle being present only during late summer and autumn; and (3) alternate grazing, cattle being present every other year (for 3–9 months). Transect counts of adult butterflies were done during the flight period of both butterfly species in the summer of 2003 and 2004. We analyzed these frequency counts by log linear models with Poisson error, testing the effects of grazing regime (four classes: continuous grazing, seasonal grazing, alternate grazing, and abandoned), sampling year (two classes: 2003 and 2004), sampling effort (transect duration in minutes), and tree/edge density (three classes: some trees and/or edges, intermediate density of trees and/or edges, high density of trees and edges) with AIC-based model selection (Burnham and Anderson 2002; Johnson and Omland 2004; Richards 2005) implemented in SAS. To take into account model selection uncertainty, we report the AIC weight of each explanatory variable, expressing the probability that the variable influences the response (here, the frequency of butterflies), the model-averaged parameter estimates and the confidence limits (Burnham and Anderson 2002).

The frequency of *P. eunomia* adults was highly related to grazing regime, sampling effort, and sampling year, as shown by an AIC weight above 98% (Table 1). *P. eunomia* adults were more numerous in control plots than in grazed plots (Fig. 3a), but much rarer in continuously grazed plots (~75% loss) than in seasonally or alternately grazed plots (~25% loss). More *P. eunomia* butterflies were counted during longer transects, more in 2004 than in 2003. The abundance of *L. helle* adults was highly related to grazing regime and tree/edge density, showing no significant differences between years, and only a marginal increase with sampling effort (Table 1). A significant difference (~50% loss) was detected in the case of seasonal grazing (Fig. 3b). When considering both species together, the impact

**Table 1** Factors affecting the adult density of the two butterfly species as observed along transect lines, as modeled using log linear models

Variable	Modality	<i>Proclissiana eunomia</i>				<i>Lycæna helle</i>			
		AIC weight (%)	Estimate	Lower	Upper	AIC weight (%)	Estimate	Lower	Upper
Grazing Type	Alternate	100.00	-0.374	-0.661	-0.088	98.57	0.431	-0.145	1.006
	Continuous		-1.041	-1.521	-0.561		-0.185	-1.457	1.086
	Seasonal		-0.510	-0.799	-0.221		-1.239	-2.112	-0.366
	Control		0.000				0.000		
Time	Time	100.00	0.020	0.008	0.032	50.84	0.025	-0.005	0.055
	Year	98.97	-1.152	-1.357	-0.947	40.59	0.470	-0.217	1.157
Tree/Edge	2004		0.000				0.000		
	Edge1	43.44	0.242	-0.153	0.636	98.61	-0.714	-1.692	0.264
	Edge2		0.084	-0.212	0.379		0.775	-0.048	1.597
	Edge3		0.000				0.000		

AIC weight expresses the probability that the variable influences the density. Parameter estimates and confidence intervals for each modality were model averaged to take model selection uncertainty into account (Burnham and Anderson 2002). Parameter estimates quantify the difference in the log of the frequency with the last modality of each variable, fixed at 0 by definition



**Fig. 3** Impact of grazing treatment on the frequency of the two butterfly species. Shown are the percentages of adult butterflies counted in grazed and neighboring control plots along transects at same dates for same durations, **(a)** for *P. eunomia*, **(b)** for *L. helle*. NB: In the case of alternate grazing (two localities), there was no strict control plot available in one locality (Deifelt); each plot being grazed for 3 months in autumn every second year, the temporarily ungrazed plot from the two adjacent plots was used as a control (in this graph only, for loss estimation – not in the log linear models of Table 1). Mean butterfly densities (measured by the number of individuals counted in 30 min) on these two Deifelt plots were very similar or higher after the start of grazing management than before, in the nineties (*P. eunomia*: 23,9 ind./30 min. after vs 16,9 ind./30 min. before; *L. helle*: 3,7 ind./30 min. after vs 3,8 ind./30 min. before), suggesting no detectable detrimental effect of this grazing regime on these species in this particular site

on populations was the least marked with alternate grazing regime, and higher with seasonal and continuous grazing (Table 1).

### 3.2 Linking the Impact of Grazing with Habitat Requirements

Impact of light grazing on the populations of *P. eunomia* and *L. helle* proved to be non-existent to moderate (<30% loss) or heavy (>50% loss) according to the grazing regime adopted and the species concerned, a periodic application generally appearing less harmful than a continuous one. This could be explained by the negative impact of livestock on (1) nectar flowers during the flight period of the butterflies and on (2) the bistort due to the trampling on rhizomes, and some direct impact on the early stages of the butterflies (Schtickzelle et al. 2007). However, butterfly losses amounting to >70%, as reported by Schtickzelle et al. (2007) on *P. eunomia* in another Ardenne site (Prés de la Lienne), were only recorded on the site with continuous grazing regime (Martelange) in the present study. They were far more reduced on the other sites where periodic grazing was practiced. The regime applied in Prés de la Lienne was also continuous, but with heavier stocking rates (comprised between 0.23 and 0.42 Livestock Units ha<sup>-1</sup> on

an annual basis), even higher during the summer (0.54–0.74 LU ha<sup>-1</sup>), compared to the sites studied here.

The influence of tree/edge density on *L. helle* counts is readily explained by several aspects of adult life in this species. Indeed, scrubs and trees, from the vicinity of which they are rarely found away (Fisher et al. 1999; Turlure et al. 2009), are used as territorial, nectaring, and roosting places. First, males exhibit a perching territorial behavior along forest edges and bushes, engaging chasing flights after males and waiting for females to mate with. Moreover, both males and females exploit flowering bushes and trees in search of nectar and higher trees for roosting: at the end of the day, they reach the top of the trees, step by step, where they usually spend the night (Goffart and Waeyenbergh 1994; Goffart et al. 2001; Goffart unpublished data). *L. helle* appears to find an optimal habitat in sites with an intermediate density of edge and trees in and around wet grasslands.

## 4 Options for Management

Open semi-natural habitats, like those inhabited by the two relict butterfly species studied here, must be managed to prevent forest recolonization. The real question of interest to people in charge of the design and implementation of biodiversity conservation plans is how to determine the management protocols that would yield the best conservation results. Mowing and grazing are currently used in many biodiversity conservation and management actions, including Ardenne humid grasslands. However, we showed here that both can have very detrimental effects on target species, if used without caution, and they must therefore not be blindly applied in any case.

Some studies showed that mown grasslands present a reduced butterfly richness and abundance compared with abandoned ones (Erhardt 1985; Erhardt and Thomas 1991; Munguira and Thomas 1992; Feber and Smith 1995, Feber et al. 1996), while other studies showed that hay meadows can harbor a rich and original fauna, including rare species, compared with other biotopes (Swengel 1996; Wettstein and Schmid 1999; Stefanescu et al. 2005). This study and other results of the same experiment (Goffart et al. 2001) suggest that the impact of mowing on populations may be highly negative, dramatically diminishing the butterfly densities (>50% loss). *P. eunomia* appears especially sensitive to mowing, with a real avoidance of mown areas by adults and a disastrous effect on early instars. The likely explanation is that this species is linked to habitats where structural elements like grass tussocks can be found (Goffart et al. 2001; Turlure et al. 2009). Its populations can be affected by a widespread application of cutting management in the presently abandoned humid grasslands. The persistence of populations in the Ardenne valleys at a time when mowing practices were widespread can probably be explained by the far bigger extent of its habitat in the past, allowing for regional persistence of populations despite the decrease in population density and/or extirpation

of local populations due to mowing. Indeed, movements of individuals from occupied sites allowed for metapopulation dynamics through rescue effect (immigrants increase population density) and recolonization (Hanski and Gilpin 1997). The non-mechanical way of cutting and its temporal spread might also have had a lower impact than current mowing techniques. Thus, the traditional way of management of these biotopes cannot be retained as an ideal solution for the long-term conservation of *P. eunomia* populations.

Light grazing, being a more “natural” way of management (although not a traditional way) and generating heterogeneity in the vegetation cover (Van Wieren 1991, 1995; WallisDeVries et al. 1998), therefore appears as a prime alternative. Indeed, several studies showed that grazing management can be very efficient to conserve butterfly populations or communities and their habitats, when used with light or moderate stocking level (Dolek and Geyer 1997; Ellingsen et al. 1997; Weiss 1999; WallisDeVries and Raemakers 2001; Ellis 2003; Pöyri et al. 2004; Öckinger et al. 2006). The present study indicates that grazing can also be a management option for *P. eunomia* and *L. helle*, the two threatened species linked to a food plant highly sensible to grazing and trampling, if the following conditions are applied: (1) very light stocking rates are used:  $<0.2 \text{ LU ha}^{-1}$ , as recommended already by others in other contexts (WallisDeVries and Raemakers 2001; Ellis 2003), (2) preferably used in a rotational basis, and (3) avoiding crucial parts of the butterfly life cycle (April to July for *P. eunomia* and *L. helle*).

However, despite its effectiveness in conserving habitats of these target butterflies with relatively limited impacts on their populations, grazing should not be considered as “the” only appropriate and adequate management option in Ardenne humid grasslands. Indeed, light grazing is difficult to apply on small areas, where the risk of overgrazing is higher (Schtickzelle et al. 2007), and this method is better applied in large areas (Bakker and Londo 1998; Ellingsen et al. 1997). Actually, the impact of grazing on butterfly abundance or richness is highly variable and clearly dependent on its intensity and the modalities of its application (Kruess and Tschardt 2002; Pöyri et al. 2006). Very detrimental effects have been reported at high stocking levels (Wettstein and Schmid 1999; Kruess and Tschardt 2002; Ellis 2003; Schtickzelle et al. 2007). Mowing may then replace grazing on small areas ( $<1 \text{ ha}$ ), if used with caution, on a rotational basis, with a pluriannual regime.

More generally, the choice of a single management method (i.e., mowing or grazing) is not advisable in a conservation perspective at the landscape scale. The use of different management practices and intensities, including permanent or temporary abandonment, can be fruitful for individual species (Schwarzwälder et al. 1997) as well as entire communities (Swengel 1998; Dolek and Geyer 1997; Wettstein and Schmid 1999; Balmer and Erhardt 2000; Pöyri et al. 2005; Vögel et al. 2007). The particular context of each site, and especially the area of the habitat in the landscape, is clearly an important parameter regarding the choice of the management method aimed at the conservation of viable butterfly populations and of a rich fauna in this kind of biotope.

## 5 Recommendations for Management and Scientific Monitoring

Applying “general recipes” may be detrimental because the impact of management is not similar in different biotopes, with different species and/or ecological conditions. We must nevertheless remember that every management act has a potential negative impact and we are probably forced to admit some minimal loss in order to achieve management actions that will in the end prove to be positive. To minimize this potential loss, our recommendations follow several major lines:

1. Scientific monitoring of the impact of management should be improved by focusing more precisely on the requirements of species in terms of resources and by considering management impact on the different life stages of the species.
2. Unmanaged areas must be preserved in any implementation of a management procedure. They may serve as a comparison basis for monitoring and assessing the effect of management and also as a refuge for populations (Johst et al. 2006; Swengel and Swengel 2007).
3. Pluriannual rotational regimes (Oates 1995; Morris 2000) that leave some areas unmanaged every year must be clearly preferred over any static management.
4. Implementation of management methods should be decided on the basis of landscape composition. We then urge scientists to take into account the impact of management on the Population Viability Analyses that serve to elaborate conservation guidelines (Schtickzelle and Baguette 2009).

**Acknowledgments** The authors would like to thank Michel Waeyenbergh, Anne Weiserbs, Gabriel Nève, Isabelle Convié, Isabelle Brasseur, Catherine Burnotte, Julie Choult, Michel Baguette, Philippe Fayt, and Michel Pirnay who helped during field work. We are very grateful to Philippe Lebrun, Michel Baguette, Hans Van Dyck, Marc Dufrêne, and Pierre Gérard, who provided support at different stages of the work and Thomas Schmitt who commented on an earlier version of the manuscript. Camille Turlure acknowledges a PhD grant from the FRIA institution. Nicolas Schtickzelle is research associate of the Fund for Scientific Research–FNRS.

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# Review

## Modelling Future Trends of Relict Species

Dennis Rödder and Johannes Dambach

**Abstract** Distribution patterns of species vary over space and time. This becomes most evident considering the differences between the current and Last Glacial Maximum (LGM, 21,000 BP) distribution patterns of species in the Northern hemisphere (see also Habel et al. 2009, Mini Review 03). Most warm-adapted species experienced reduction and fragmentation of ranges because of intrusion by uninhabitable continental ice sheets causing distributional shifts and fragmentation of primary habitats. On the other hand, cold-adapted species were able to expand their ranges. Today, ranges of those species are restricted to current refugia as can be observed in glacial relict species.

Understanding refugial distributions of species has been a core task in historical bio-geography. Before the 1990s, refugia were preliminarily identified based on disjunctions of species distributions, distribution patterns of sister species, and the fossil records (e.g. Hoffman 1981). More recently, phylogeographic approaches based on intraspecific molecular analyses and spatial modeling approaches based on ecological properties of species have been developed.

### 1 Climatic Niches and the Spatial Distribution of Species

The observation of ecological properties of species and their areas of distribution being related is not new (Grinnell 1917; James et al. 1984), but the increasing availability of information on the variation of environmental parameters in geographic

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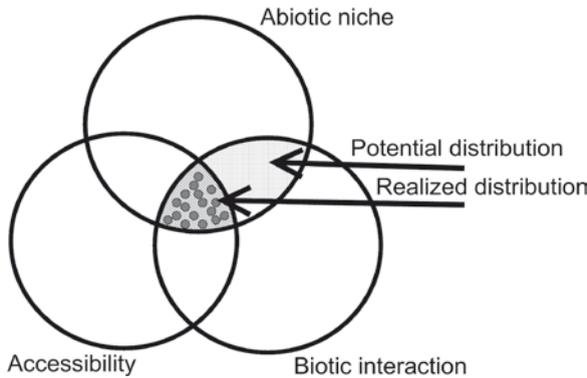
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space, species distribution data, and computation capacities during the last decade now allow large scale assessments of relationships between distributions observed and explanatory parameters. Relationships can be assessed by calculating ‘environmental’ or ‘ecological’ niches and their subsequent projection into geographic space (Guisan and Zimmermann 2000). Here, GIS-based environmental data offer huge opportunities to assess variations in environmental factors within the species ranges, especially when combined with spatial modeling techniques (Kozak et al. 2008; Waltari et al. 2007). Such techniques were proposed as a useful supplementary tool despite long time/term established techniques for the identification of refuges and potential migration pathways (Waltari et al. 2007).

Model techniques can be classified into two different groups: (1) mechanistic models, which predict the potential distribution of a species based on its physiological tolerances measured in laboratory experiments and (2) spatial model techniques, which derive from the distribution model based on statistical relationships between distribution patterns observed and environmental parameters. In the latter case, Climatic Envelope Models (CEMs) use exclusively climatic variables as predictors whereby a wider range of variables is used in Ecological Niche Models (ENMs) (e.g. soil and vegetation layers or remote sensing data). The development of mechanistic models is just at the beginning (Kearney and Porter 2004; Kearney et al. 2008), but CEMs and ENMs have been applied to predict species’ potential distributions (PDs) under current, past, and future climate scenarios for some time now (e.g. Araújo et al. 2004; Araújo and Guisan 2006; Heikkinen et al. 2006; Hijmans and Graham 2006; Pearman et al. 2008; Waltari et al. 2007), invasive species biology (e.g. Peterson 2003; Peterson and Vieglais 2001; Rödder 2009; Rödder et al. 2008), conservation priority setting (e.g. Araújo et al. 2004; Kremen et al. 2008), and ecology and evolutionary biology (e.g. Graham et al. 2004; Kozak et al. 2008; Peterson et al. 1999). CEMs were especially suggested to be useful for identification of potential Pleistocene refugia with high accuracy (Peterson and Nyári 2008; Waltari et al. 2007) and are therefore discussed below in detail.

In CEM approaches, the climate envelope is understood as a part of a species’ fundamental or realized niche depending on variables selected and assumptions made (Soberón 2007; Soberón and Peterson 2005). As defined by Hutchinson (1957; 1978) and later extended by Soberón and Peterson (2005), a species’ fundamental niche represents the complete set of environmental conditions under which a species can persist, i.e. under which its fitness is greater than or equal to one in the absence of competitors or predators. Its realized niche in environmental space (=realized distribution in geographic space) is a subset of the fundamental niche considering dispersal limitations and biotic interactions, such as food availability, competition, or interactions with pathogens (Fig. 1). Niche variables can be subdivided concerning specific classes regarding the spatial extent in which they operate and if competition may play a role or not. The Grinnellian class is defined by fundamentally non-interactive variables (e.g. climate) (Grinnell 1917) whereby the Eltonian class focuses on biotic interactions and resource-consumer dynamics (Elton 1927). The former operates on a coarse scale and is the main subject in CEM approaches, whereby the latter can principally be measured only at local scales and is commonly not addressed in CEM approaches (Soberón 2007).



**Fig. 1** Relationships between abiotic (=fundamental) niche, biotic interaction and accessibility after Hutchinson (1957) as modified by Soberón and Peterson (2005). The potential distribution is a subset of the abiotic niche considering biotic interactions, whereby the realized distribution is a subset of the potential distribution considering accessibility. *Dots* represent species records

## 2 How do CEMs Work?

Before CEMs can be computed it is necessary to compile a set of species occurrence records and a set of suitable predictor variables (e.g. GIS layers containing information on climatic parameters). Some examples of freely available climate data sets are given in Kozak et al. 2008; Rödder et al. 2008, Chap. 22. The layers for each climate scenario commonly include the minimum and maximum temperatures and the mean precipitation per month (= 36 climatic parameters). Based on these monthly layers, bioclimatic parameters can be generated, e.g. with DIVA-GIS (Hijmans et al. 2001). Bioclimatic parameters are more useful than monthly values, since they are independent of latitudinal variations. This becomes obvious considering that the ‘maximum temperature of the warmest month’ is more useful than the maximum temperature of a specific month, since not everywhere the same month might also be the warmest.

Multi-collinearity among predictor variables may hamper the analysis of species-environment relationships because ecologically more causal variables may be excluded from models if other correlated variables explain the variation in response variable better in statistical terms (Heikkinen et al. 2006). Therefore, variable selection should be guided by a throughout assessment of the target species’ ecology, and a rather minimalist set of predictors should be preferred. Specific adjustment of variable sets according to specific ecological needs of the target species may improve the model output.

Once species records and predictor variables have been compiled, a subsequent step involves the development of a multidimensional view of the climatic niche of a species, which is a considerable challenge given the complex nature of species’ niches (Peterson and Vargas 1993). In plain text: in CEM, climatic information for species presence localities are summarized to an ‘ideal’ climatic niche for the target

species (note that this can also be done with reliable absence data) that is afterwards compared to climatic conditions at the query localities, i.e. where the presence/absence of the species is unknown. The results are geographic maps showing the similarity of an area with the 'ideal' climatic niche (Figs. 2a, and 3a). The selection of a suitable algorithm for the computation of the CEM depends on the amount of distribution records available, their quality, and the specific goal of the study (for a brief overview of available algorithms see Jeschke and Strayer 2008 or Rödder et al. 2008, Chap. 22).

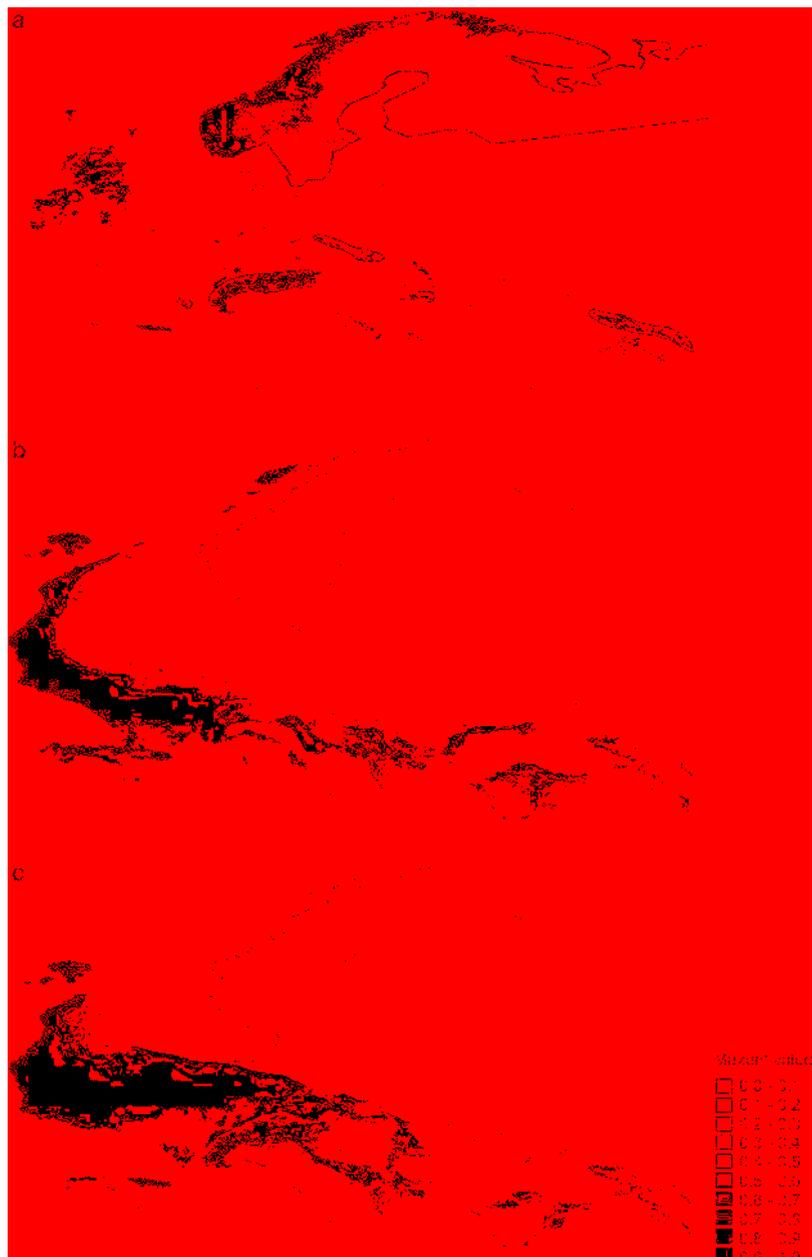
### 3 Evaluation of Results

One of the most frequently used model tests is an assessment of the Area Under the Curve (AUC) statistics, referring to the ROC (Receiver Operation Characteristic) curve using e.g. 25 % of the records as test points and the remaining ones for training (Hanley and McNeil 1982; Phillips et al. 2006). This method is recommended for ecological applications because it is non-parametric (Pearce and Ferrier 2000). Values of AUC range from 0.5 (i.e. random) for models with no predictive ability to 1.0 for models giving perfect predictions. According to the classification of Swets (1988) AUC values  $>0.9$  describe 'very good',  $>0.8$  'good' and  $>0.7$  'useful' discrimination ability. A second possibility is Cohen's kappa statistic of similarity ( $k$ ) (Fielding and Bell 1997; Pearce and Ferrier 2000). Cohen's kappa yields values ranging from 0.0 (no predictive ability) to 1.0 (perfect predictive ability), whereby  $k$  values above 0.7 describe 'very good' discrimination ability (Monserud and Leemans 1992). If only few species records for model building are available (e.g.  $<25$ ), a jack-knife test can be performed (Pearson et al. 2007). Additionally, in some cases invasive populations of the target species can be used as an independent test (Sax et al. 2008).

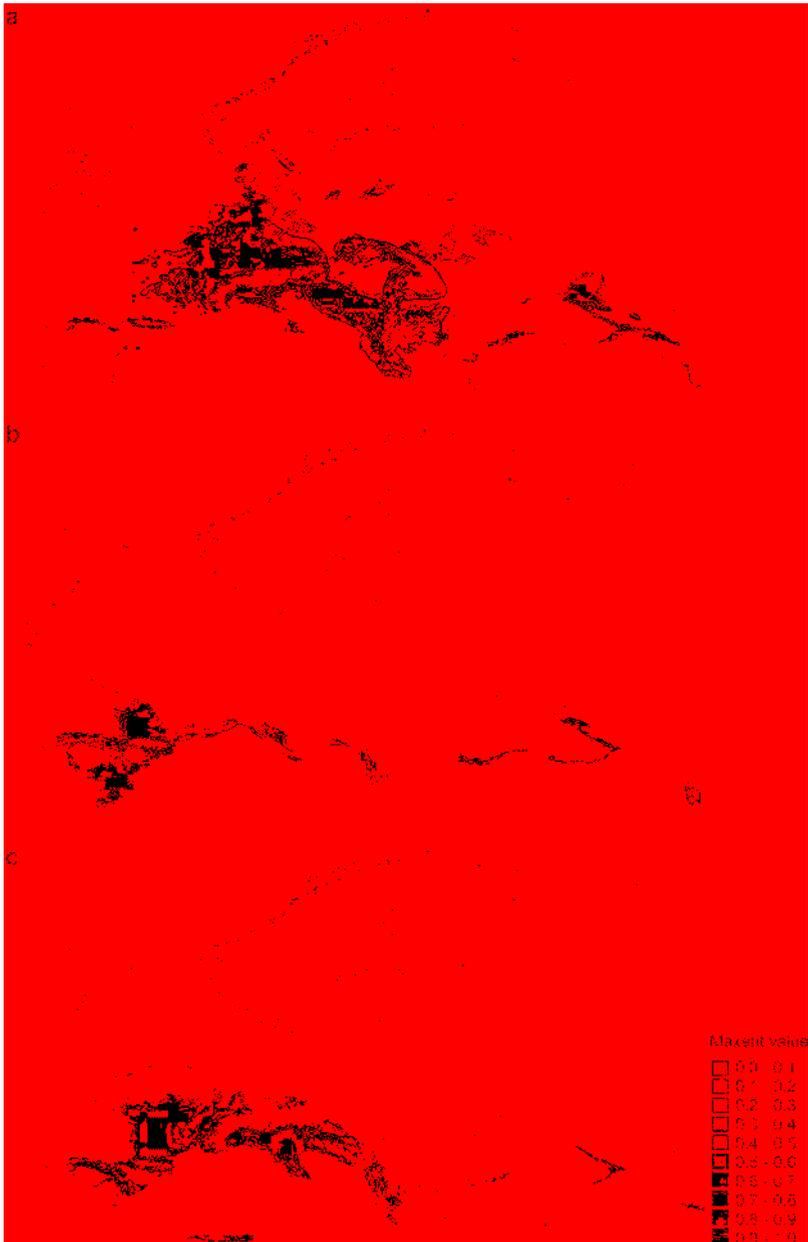
It needs to be noted that test statistics, such as AUC or Cohen's kappa scores are a measure of model fit to be compared to the observed distribution of a species and must not be interpreted as a measure in the ecological sense of a model. Profound knowledge of a species' natural history is necessary for an evaluation, when a model meets the ecological requirements of the target species.

### 4 Strengths of CEMs and Potential 'Pitfalls' When Interpreting Results

CEMs are easy to use and inexpensive. A steadily increasing number of studies have been showing that, when CEMs are properly applied, results can have a high quality (e.g. Peterson 2003; Waltari et al. 2007). They have been a rich source of quantitative projections concerning geographic ranges of species with great value in many areas of both basic and applied ecology and conservation. They allow the



**Fig. 2** Today's distribution of *Aeshna caerulea* (a) hatched areas, source: Kuhn and Burbach (1998), Sternberg and Buchwald (2000) and its potential distribution computed with Maxent 3.2.1 derived from current climatic conditions. Higher Maxent values suggest higher climatic suitability. Figure 2b,c show potential distributions of *Aeshna caerulea* assuming two different paleoclimatic scenarios depicting climatic conditions as expected for 21,000 BP (b) CCSM; 2C: MIROC; for details see Rödder et al. 2008, Chap. 22. Areas of currently known distribution of *A. caerulea* are highly congruent with the proposed potential distribution of the CEM even in small and disjunctive ranges. Projections of the CEM onto palaeoclimatic scenarios suggest potential migration pathways during the LGM connecting most current refugia



**Fig. 3** Today's distribution of *Bombina variegata* (a) hatched areas, source: IUCN (2006) and its potential distribution computed with Maxent 3.2.1 derived from current climatic conditions. Higher Maxent values suggest higher climatic suitability. Figure 3b,c show projections of the climate envelope of *B. variegata* assuming two different paleoclimatic scenarios depicting climatic conditions as expected for 21,000 BP (b) CCSM; 3C: MIROC; for details see Rödder et al. (2009), Chap. 22. Areas of currently known distribution of *B. variegata* are highly congruent with the proposed potential distribution of the CEM even in small and disjunctive ranges, e.g. in France. Projections of the CEM onto palaeoclimatic scenarios suggest potential refugia during the LGM

identification of possible threats due to climate change (e.g. see Habel et al. 2009, Chap. 25) or past migration pathways (Fig. 2b, c, Rödder 2009, Chap. 22).

For example, CEM projection of the PD of the Azure Hawker (*Aeshna caerulea* (Stroem, 1783)) onto palaeo-climatic scenarios reflecting conditions as expected for the LGM 21,000 BP suggest that climatically suitable areas were widely distributed in Europe throughout the LGM (Fig. 2b,c). The PD would have connected the current refugia (Fig. 2a). It is also possible to reconstruct the past refugia of a currently wide-spread species (Waltari et al. 2007), e.g. as shown for the yellow-bellied toad (Fig. 3). Furthermore, CEMs can be used as a guideline for efficient further sampling, since phylogeographic studies are expensive in terms of both time and resources. Regarding relict species, CEMs may be useful for the identification of potentially suitable areas which may harbor relict populations unknown so far.

However, there are some points that deserve consideration when interpreting results, since a number of potential sources of predictive errors that may be categorized as either 'algorithmic' or 'biotic' do exist (Fielding and Bell 1997). Algorithmic errors are caused by limitations of the modeling technique and data-gathering process. Each algorithm makes specific assumptions and therefore has strengths and weaknesses, e.g. some algorithms are able to account for interactions between predictor variables whereas others are not. This influences their predictive ability and suitability in specific cases. Differences between algorithms need to be evaluated regarding their usefulness depending on the study goal at hand. When projecting CEMs onto past or future climate scenarios it is important to acknowledge that different climate scenarios will reveal different results and no single 'best' model exists. "Strengths and weaknesses" characteristics/qualities of different climate models should be taken into consideration (Beaumont et al. 2008). Evaluation of a variety of scenarios may help to assess output variations. For example, when comparing Figs. 2b,c and 3b,c. the general patterns are similar, but fine scale differences, especially in Northward PD limits, can be identified. These can be traced back to different assumptions made in the palaeo-climatic scenarios used, e.g. concerning CO<sub>2</sub> concentrations.

CEMs are derived from a subset of environmental conditions at species records (= variables selected); hence they capture only a more or less extensive part of the climatic niche depending on the variables selected and other factors influencing the distribution of species (see below). They are generally unable to capture the niche completely, and output maps show regions with similar conditions as the testing records according to the predictor variables selected rather than 'complete' niches.

Quality and spatial properties of distribution records used for model building can significantly influence the results, e.g. when samples do not cover the whole climatic niche space of the target species. Furthermore, some algorithms require that samples are independently distributed of each other, which is seldom the case in biological data. Therefore, spatial autocorrelation – the tendency of neighboring samples to possess similar characteristics – is a potential problem in many niche modeling approaches and several methods to account for this fact have been suggested (Dormann et al. 2007).

When applying CEMs some key assumptions regarding biological aspects are commonly made: (1) species occur at all locations where the climate is favorable,

(2) biotic interactions are unimportant in determining ranges and are constant over space and time and (3) genetic and phenotypic compositions of species are constant over space and time (Jeschke and Strayer 2008). (1) Ideally CEMs highlight all areas that are climatically suitable for the target species reflecting its potential distribution regardless of dispersal limitations and thus accessibility. When interpreting potential distribution maps it is important to evaluate the dispersal properties of the target species. For example, although *Bombina variegata* (Linnaeus, 1758) could find climatically suitable areas in England today (Fig. 3a), the species is unable to colonize the UK due to dispersal limitation by the sea. (2) Biotic interactions are commonly not considered during model building since interactions, such as competition or predation in species assemblages are often too complex to be gathered in a single step during model building. For example, the PD of *B. variegata* is considerably wider than its realized distribution in Germany, the Czech Republic, Slovakia and Hungary (Fig. 3a). Here, climate is not the limiting factor since a hybrid-zone between *B. variegata* and its sister species *Bombina bombina* (Linnaeus 1761) hamper further dispersal (Szymura 1993). Under different climate scenarios, PDs of species can respond spatially in a different manner which can cause strong variations over space and time and result in discrepancies (e.g. as shown by Schweiger et al. (2008) in an ENM approach for a butterfly / host plant system). For simplicity's purpose it may be useful to focus in a first step on the climatic niches of each target species to assess its potential distribution and subsequently compare models for potentially interacting species. (3) An important point when projecting CEMs onto past or future climate scenarios is that fundamental niches can be subject to evolution. In a recent review, it has been shown that, independent of the taxonomic group, the fundamental niche can remain stable for tens of thousands of years or it can shift substantially within only a few generations (Pearman et al. 2007). 'Niche conservatism' of closely related species is a phenomenon that has been observed in several different taxonomic groups (e.g., Peterson et al. 1999; Wiens and Graham 2005), but, on the other hand, niche shifts have also been proposed (e.g. Broennimann et al. 2007; Fitzpatrick et al. 2007; Graham et al. 2004; for a recent review see Pearman et al. 2007). So evidence for the degree of niche shifts or niche conservatism in closely related species is mixed and debated (Pearman et al. 2007; Wiens and Graham 2005). When applying CEMs, it is an important issue if (climatic) niches are rather conservative or not, especially when the aim is an assessment of changes in potential distributions under different climate scenarios. However, in general, there is a considerable lack of knowledge regarding the processes triggering shift in climatic niches as well as in suitable methods to analyze it.

## 5 Conclusions

Predictions of species distributions derived from correlative models can help to understand the spatial patterns of biodiversity, refugia and identification of possible threats for relict populations. The amount of available data and software is steadily

increasing as well as the number of studies applying niche model techniques. However, a discrepancy between increasingly complex studies and the understanding of underlying processes, derivation of valid assumptions, and the development of conceptual backgrounds is still a problem (Jiménez-Valverde et al. 2008). One problem might be that models predicting potential distributions of species are easily computed and that test statistics are sometimes misleading (Lobo et al. 2008) facilitating misinterpretations. However, properly applied and critically evaluated, during each step, from model building to the final interpretation, CEMs can be powerful tools and very helpful to generate and test hypotheses.

**Acknowledgements** We are indebted to Bernhard Misof and Stefan Lötters for fruitful discussions and Michael Raupach for logistic support. Robert Hijmans helped us with palaeoclimatic scenarios. DR was funded by the ‘Graduiertenförderung des Landes Nordrhein-Westfalen’ and JD by the ‘Deutsche Forschungsgemeinschaft’ (DFG).

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# Conservation of the Grey Bush Cricket *Platycleis albopunctata* (Orthoptera: Tettigoniidae) Under Differing Habitat Conditions: Implications From an Individual-Based Model

Eva M. Griebeler and Eckhard Gottschalk

**Abstract** Assessing the chance of survival of any species is a great challenge in conservation biology. In this chapter, we analyse the vulnerability of the grey bush cricket *Platycleis albopunctata* in habitats of different food availability under current and increased temperature conditions applying an individual-based model. Our simulations show that populations in warmer habitats with a higher food limitation have a much lower extinction risk than those living in habitats that are less food-limited and colder. An increase in mortalities of life stages severely increases the risk of population extinction, whereas a shift in the termination of egg diapause towards the beginning of the year caused by warmer winters has no significant effect on survival. Thus, *P. albopunctata* may benefit from increasing future temperatures if neither life stage mortalities will increase as the result of simultaneously affected abiotic habitat conditions (due to precipitation and temperature) and/or species interactions nor changes in abiotic conditions will cause a shortage of food. We demonstrate that simulation models are a suitable tool to understand processes influencing population survival of temperature-dependent species under current and future habitat conditions. They are in particular beneficial in conserving relict species that are naturally restricted to patchy and scarce habitats.

## 1 Introduction

Orthopteran insect species are a major taxonomic group in natural and disturbed ecosystems of temperate and subtropical Eurasia (Sergeev 1998). These species occur in both natural (particularly grasslands) and anthropogenic landscapes

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(meadows, pastures, agricultural fields, etc.). They are very sensitive to temperature, are especially characteristic of hot and sunny regions and are in general no food specialists. Populations of grasshoppers and bush crickets are declining in Central Europe (Ingrisch and Köhler 1998). More than half of these species are already considered endangered since many of their habitats are rapidly being destroyed, fragmented and changed (Ingrisch and Köhler 1998). However, over the past decade there has been increasing evidence that several orthopterans are able to shift their ranges towards the North as a consequence of present global warming (Kleukers et al. 1996; Cannon 1998; Widgery 2000).

Small population sizes are often the consequence of recent habitat fragmentation or loss. However, they also characterize highly ecologically specialized organisms restricted to scarce habitats such as relict species. Such species show highly localized ranges and are pre-dominantly found in regions where past climate changes have not resulted in major extinction events. Although it is widely acknowledged that population persistence greatly depends on population size and temporal fluctuations, the long historical persistence of many relict and endemic taxa as small populations contradicts this relationship. However, anthropogenic habitat fragmentation, alteration and loss are threats to any species. Thus, assessing the chance of species survival under current and future environmental conditions is especially important for species inhabiting patchy and scarce habitats.

The thermophilic grey bush cricket *Platycleis albopunctata* (Orthoptera: Tettigoniidae) is distributed throughout Europe. Because dry grassland has a patchy distribution in Central Europe, populations of this cricket inhabit habitat islands. During the last century, habitat fragmentation has increased due to changes in land use. The species is considered threatened in Germany (category three, Ingrisch and Köhler 1998) because of the ongoing loss of dry grasslands which results in relict populations. Over the past decades, there has been increasing evidence that *P. albopunctata* is shifting its range towards the North as the result of present global warming (Widgery 2000). Field studies on this cricket have shown that temperature conditions and vegetation density of habitats strongly differ (Gottschalk et al. 2003). It has been suggested that such differences in habitat conditions affect population dynamics of this species and that this may consequently have an impact on its extinction risk (Gottschalk et al. 2003). Although warmer habitats (e.g. dry grasslands) allow for higher reproduction rates and faster development of life stages (Köhler 1999), they have sparser vegetation than colder habitats (e.g. semi-dry grasslands) and a lower rate of primary production which causes food limitation.

In order to better understand the mechanisms that determine the vulnerability of this cricket under current and future thermal conditions in habitats of different food availability, we utilized an individual-based model (IBM) from a previous publication (Griebeler and Gottschalk 2000a). IBMs have been successfully applied to various species (DeAngelis and Mooij 2005) including orthopterans (Wagner and Berger 1996; Griebeler and Gottschalk 2000a,b) to assess their degree of endangering. We test the hypothesis of differing population dynamics generated by differences

in temperature conditions and in food availability that was proposed by Gottschalk et al. (2003) based on field observations and laboratory experiments. Therefore, we apply it to the two populations experimentally studied in this paper (in contrast to Griebeler and Gottschalk 2000a,b; Gottschalk et al. 2003), and additionally consider a broader number of habitat conditions by varying temperature profiles and food availability of habitats. Furthermore, we perform a sensitivity analysis for mortalities of life stages and the length of egg diapause, as these may also be altered under global change as a result of simultaneously affected abiotic conditions and/or species interactions.

## 2 Materials and Methods

### 2.1 *P. albopunctata*

The grey bush cricket *P. a. albopunctata* (Orthoptera: Tettigoniidae) is distributed throughout West and Central Europe (Harz 1969). Its sister subspecies *P. a. grisea* extends its distribution area into Asia. *P. a. albopunctata* has an Atlantic-Mediterranean origin, whereas *P. a. grisea* originates from Central Asia (Ingrisch and Köhler 1998).

*P. albopunctata* is a thermo- and xerothermophilic species (Harz 1957; Ingrisch and Köhler 1998). In Central Europe, it inhabits dry grasslands and similarly structured semi-arid and arid habitats (Harz 1957). Only in habitats with favourable microclimatic conditions is development of instars sufficiently rapid to provide a reproductive output that enables long-term survival (Gottschalk 1996). In Germany, the species is considered threatened due to the loss of its semi-natural habitats (Ingrisch and Köhler 1998; Walter and Gottschalk 1998). Abandonment of grazing as well as afforestation of dry grasslands has led to habitat loss resulting in relict populations within its distribution area. The life cycle of the species is annual with one generation per year (Ingrisch 1986). Growth, development and reproduction of the cricket are primarily affected by temperature (Ingrisch 1986). Under the environmental conditions in our study area (see below), hatching occurs in spring (mainly April). The reproduction period of the cricket lasts from July until temperatures decline in October (Gottschalk et al. 2003). Females produce eggs throughout their adult life. They prefer soil and moss at places with sparse vegetation as oviposition sites. Eggs develop during late summer and in the following spring after an intermediate diapause. The proportion of embryonic development that is completed before hibernation depends on the date of oviposition and ground temperature (Gottschalk et al. 2003). Egg hatching after hibernation needs no frost stimulus, but larvae need higher temperatures to hatch (Ingrisch 1985). The first instars of *P. albopunctata* mainly feed on flowers (anthers, nectar glands, petals). Older nymphs and adults prefer seeds, especially from grasses, and adults also feed on small insects. Both sexes are able to fly.

## 2.2 *Field Studies and Summary of the Field Experiments*

Our two study sites are situated about 50 km apart from each other in North Bavaria (southern Germany, 48°N and 10°E). The size of each of the areas is about 1 ha. The “Hammelburg” area is a dry grassland located in the valley of the Franconian Saale on the Southern edge of the Rhön mountains at an altitude of about 280 m. The area is a steep slope that is exposed to the Southwest with an inclination of 35°. The “Prappach” area is a semi-dry grassland in the Main valley at the edge of the Hassberge. This slope is situated at an altitude of 270 m. The area consists of a flat plateau and several steeper embankments with inclinations ranging between 0 and 20°. The area is irregularly grazed by sheep or mowed. Vegetation cover is lower at Hammelburg (median 50%, Gottschalk et al. 2003) than at Prappach (median 80%, Gottschalk et al. 2003). The number of seeds on the ground as a measure for productivity is six-fold higher in Prappach, and the average distance to the next potential food plants is ten-fold lower in Prappach than in Hammelburg (Gottschalk et al. 2003). This suggests that food is more limited at Hammelburg than at Prappach.

In 2002 we performed measurements of ground temperature in both areas between 8th August and 9th September, because this period between time of oviposition and the autumn temperature decline is important for embryonic development (Ingrisch 1979; Gottschalk et al. 2003). The measurements revealed that Hammelburg is on average 4.8°C warmer than Prappach (Gottschalk et al. 2003).

Population abundances estimated from standardized transect walks between 1993 and 1999 showed severely differing population dynamics at both sites. Whereas Prappach exhibited strong fluctuations in population abundance (between 85 ( $\pm 20$ ) and 3188 ( $\pm 367$ ) adult animals, mean  $\pm$  standard deviation, Gottschalk et al. 2003), population sizes at Hammelburg were comparatively constant (between 460 ( $\pm 78$ ) and 730 ( $\pm 124$ ) adults, mean  $\pm$  standard deviation, Gottschalk et al. 2003). The mean population size estimated between 1993 and 1999 was 1,390 adults at Prappach and 614 adults at Hammelburg. However, in 1996 population size at Hammelburg was not estimated because of logistical limitations.

## 2.3 *The Model*

A detailed description of the model applied to test our hypothesis (Gottschalk et al. 2003) and to rate the impact of global change on the viability of *P. albopunctata* can be found in Griebeler and Gottschalk (2000a). The following is a short summary of this individual-based day-degree model.

We distinguish three life stages in this model: eggs, larvae and adults. We assume an equal sex ratio. The model is based on the idea that eggs and larvae accumulate temperatures on a daily basis during their development. Both stages need minimum temperatures for daily development ( $T_{0,\text{egg}} = 7.5^\circ\text{C}$ , Ingrisch 1986;

$T_{0,larva} = 9.6^{\circ}\text{C}$ ) and a total temperature input ( $TS_{egg} = 808.5^{\circ}\text{C days}$ , Ingrisch 1986;  $TS_{larva} = 381.9^{\circ}\text{C days}$ ) to enter the next life stage. The model considers a fixed diapause  $D$  for eggs between 1st November and 31st March of the following year (Ingrisch 1986). Eggs do not accumulate daily temperatures within these winter months. The model assumes a constant mortality rate for the egg stage ( $m_{egg} = 60\%$ ) and for the larva stage ( $m_{larva} = 90\%$ ), which summarize all mortalities during the respective life stage. These two rates that determine the final transition of a life stage to the next are randomly applied to each individual before it enters the next life stage. Each adult individual, however, has a constant daily mortality risk ( $m_{imago} = 5.1\%$ ) that is independent of its age. The daily oviposition rate of adults depends on temperature ( $Eggs(T) = 0.1 \times (10.1 \times T - 66.1)$ ,  $T$  daily temperature, Waltert et al. 1999) and egg laying requires temperatures higher than  $T_{0,egg}$ . The total reproductive output of an individual is limited ( $max_{egg} = 100$ ) and adults start reproduction at a minimal age ( $A_{min} = 14$  days).

The quality of the area inhabited by the population is modelled by two entities: (1) a constant carrying capacity  $K$  that mimics food availability and limits the daily number of adults in the population; and (2) a temperature profile that affects all life stages on each simulated day. For density regulation of the population we assume a ceiling model. Surplus adults die if an overflow in capacity occurs. They are randomly removed from the population. We use a long-term meteorological data record from the weather station “Bamberg” to model the effects of daily temperatures on population development (Griebeler and Gottschalk 2000a). This weather station is located in a distance of about 25 km from both study areas. At each simulated day, a temperature value is sequentially taken from a database that contains the daily mean temperatures registered for this meteorological station.

## 2.4 Computer Simulations

We performed three major simulation studies. In the first study, we tested whether differences in temperature conditions and food limitation account for the observed differences in population development between Prappach and Hammelburg. Therefore, by applying our model we generated population dynamics for each area over the field period between 1993 and 1999. Concerning temperature conditions, we directly applied the daily mean temperatures of the meteorological station Bamberg recorded between 1993 and 1999 for Prappach (Griebeler and Gottschalk 2000a). For Hammelburg, we added on each simulated day  $4.8^{\circ}\text{C}$  to the value obtained from this database reflecting the higher temperatures found in our field studies (Gottschalk et al. 2003). For food limitation, we assumed different carrying capacities. Capacities implementing different levels of food availability were 100, 200, 300, 400, 500, 750, 1,000, 2,000, 3,000, 4,000, 5,000, 7,500 and 10,000 adults per day. All simulations were randomly started for Prappach on 26th July 1993 with

1,218, 1,424, or 1,630 adults or for Hammelburg on 24th July 1993 with 598, 676, or 754 adults. These initial population sizes were chosen according to the population sizes estimated on these dates in the field and the respective estimation errors of sizes (Prappach:  $\pm 206$ , Hammelburg:  $\pm 78$ ). For each of the capacities considered per study area, we repeated the Monte Carlo simulation of population size 3,000 (=3 initial sizes  $\times$  1,000 simulations) times. In total, we investigated 26 (=2 areas  $\times$  13 capacities) simulation scenarios. For each of these simulation scenarios, we annually recorded population sizes in each simulation run.

To derive a more general understanding of how thermal conditions and food availability affect populations of the grey bush cricket and to rate the impact of global warming on this species, we performed a second simulation study. With this study, we aimed to estimate the extinction risk of populations inhabiting areas of different food availability with different temperature profiles. Starting with temperature conditions as observed at Prappach, we added on each simulated day 2, 4, 4.8°C (Hammelburg), 6.8 or 8.8°C to the respective temperature value of the meteorological database. For all temperature profiles we considered 15 carrying capacities (100, 250, 500, 750, 1,000, 2,000, 3,000, 4,000, 5,000, 7,500, 10,000, 15,000, 20,000, 25,000 and 30,000) implementing food availability. All 90 simulation scenarios (=6 temperature profiles  $\times$  15 capacities) were started on 1st July. The initial population consisted of adults and its size equalled the respective carrying capacity. For the annual temperature course, we applied mean daily temperatures of the meteorological station Bamberg from 1949 to 1985. For each simulation scenario, we estimated the extinction probability and the mean number of adults based on 1,000 Monte Carlo simulations over 35 years (Griebeler and Gottschalk 2000a). From these estimates, we derived minimum viable population sizes (MVP) for each temperature scenario according to Shaffer (1987). If we assume an extinction probability of 2% for a 35-year time span, this MVP definition conforms to Shaffer's (1987) limit of 5% in 100 years.

In the last simulation study (Table 1), we performed a standard sensitivity analysis for each of the mortalities assumed for life stages ( $m_{\text{egg}}$ ,  $m_{\text{larva}}$ ,  $m_{\text{adult}}$ ) and the assumed end of egg diapause ( $D_{\text{end}}$ ). We aimed to rate their individual impact on both the extinction probability and the mean annual number of adults over 35 years. Changes in habitat temperature conditions expected under global warming are likely to affect mortalities (e.g. by altering the probability at which the cricket is predated) or the termination of diapause (because eggs need no frost as stimulus for hatching and larvae hatch earlier at higher temperatures, Ingrisch 1985). Simulations were performed as in the second simulation study, with the exception that we considered only the temperature conditions of Prappach and Hammelburg. Initial population sizes and capacities were 30,000 adults for Prappach and 3,000 adults for Hammelburg, because we expected low extinction rates for these capacities and default values of mortalities and egg diapause (see Results, Table 1). Again for each simulation scenario, we estimated the extinction probability and the mean annual number of adults based on 1,000 Monte Carlo simulations over 35 years.

**Table 1** Mortalities ( $m_{\text{egg}}$ ,  $m_{\text{larva}}$ ,  $m_{\text{adult}}$ ) and ends of egg diapause ( $D_{\text{end}}$ ) studied in the third simulation study

Parameter	
$m_{\text{egg}}$	{0, 0.1, 0.2, 0.3, 0.4, 0.5, <b>0.6</b> , 0.7, 0.8, 0.9, 1}
$m_{\text{larva}}$	{0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, <b>0.9</b> , 0.925, 0.95, 0.975, 1}
$m_{\text{adult}}$	{0, 0.01, 0.02, 0.03, 0.04, <b>0.051</b> , 0.06, 0.07, 0.08, 0.09, 0.1, 0.15, 0.2}
$D_{\text{end}}$	{ <b>0</b> , 1, 2, 3, 4, 5, 6} weeks before 31st March

Bold values are defaults assumed in the model of Griebeler and Gottschalk (2000a)

### 3 Results

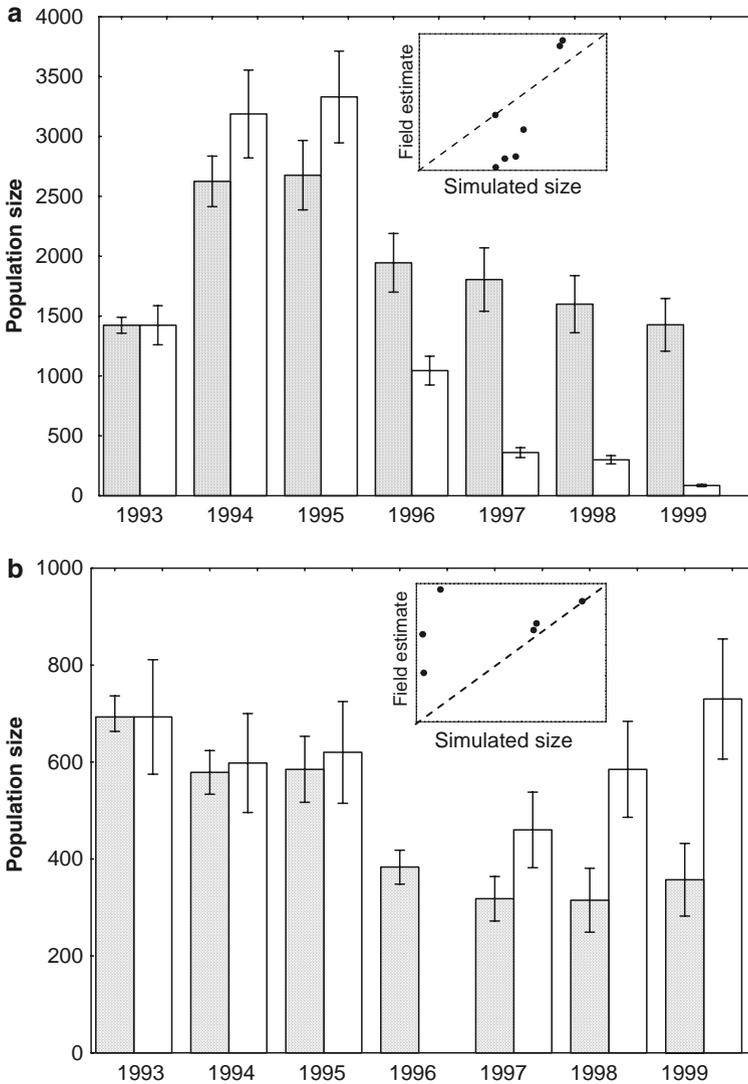
#### 3.1 Population Dynamics at Prappach and Hammelburg Between 1993 and 1999

The trend in annual population size estimated by our model for Prappach was in overall agreement with field data when carrying capacities between 1,000 and 10,000 adults were assumed (Spearman rank correlation  $r_s$  between observed and predicted sizes:  $r_s=0.786$ ,  $N=7$ ,  $p=0.05$  for all capacities between 1,000 and 10,000, Fig. 1). Population sizes steadily increased from 1993 until 1995, and the maximum size was reached in 1995. In the following 4 years, the sizes steadily decreased again, but the population never went extinct. The decline between 1995 and 1999 was much weaker in the simulations than observed in the field. Contrary to the natural population, the population frequently went extinct within the simulated time period for carrying capacities ( $K$ ) below 1,000 adults (extinction probabilities  $p_{\text{ext}}$ ;  $K \leq 400$ :  $p_{\text{ext}} = 1$ ,  $K = 500$ :  $p_{\text{ext}} = 0.644$ ,  $K = 750$ :  $p_{\text{ext}} = 0.216$ ).

The rather constant population sizes observed at Hammelburg were only reproduced by the model for capacities between 200 and 500 adults (marginal significant Spearman rank correlation  $r_s$  between observed and predicted sizes due to the smaller number of years, Sachs 1992:  $r_s=0.600$ ,  $N=6$ ,  $p=0.15$  for capacities between 200 and 500 adults, Fig. 1). The simulated course in population size became similar to the course observed at Prappach for capacities higher than 500 adults. For these capacities, a maximal size was observed in 1995 (Fig. 1). However, the population always went extinct for the lowest capacity studied ( $K = 100$ ).

#### 3.2 Effect of Temperature Conditions on the Extinction Risk of the Species

Warmer temperature conditions resulted in lower extinction probabilities of populations, higher mean annual numbers of adults and lower capacities for MVP values. The decrease in extinction probability ( $p_{\text{ext}}$ ) with increasing carrying capacities ( $K$ ) followed Hanski's (1994) model ( $p_{\text{ext}}(K) = eK^{-b}$ , where  $e \in [0, 1]$  and  $b \in [0, \infty]$ ), for all temperature profiles:  $R^2 \geq 0.972$ ,  $N = 15$ ) for all temperature profiles studied,



**Fig. 1** Simulated population sizes (*dotted bars*) and field estimates (*open bars*) at Prappach (**a**) and Hammelburg (**b**) as well as correlations between simulated sizes and field estimates (*smaller plots*) for each study area. In 1996, population size at Hammelburg was not estimated because of logistical limitations, but the population was not extinct. The carrying capacities assumed in the simulations were 10,000 adults for Prappach and 300 adults for Hammelburg. The error bars give standard deviations of the mean population size estimated in field and in 3,000 Monte Carlo simulations, respectively

whereas the mean annual number of adults linearly increased with increasing capacities for all temperature profiles (for all temperature profiles:  $R^2 \geq 0.999$ ,  $N = 15$ ). The minimum viable population sizes obtained for each temperature profile (the mean annual number of adults observed for the capacity that resulted in an extinc-

tion probability of 2%) exponentially decreased with increasing temperature conditions ( $R^2=0.993$ ,  $N=6$ ). The minimum viable population size was found for  $K=30,000$  adults (mean annual number of adults=8,341) under temperature conditions as observed at Prappach and for  $K=3,000$  adults (mean annual number of adults=1,828) under temperature conditions as at Hammelburg.

### 3.3 *Effect of Mortalities and Shifts in the Termination of Egg Diapause*

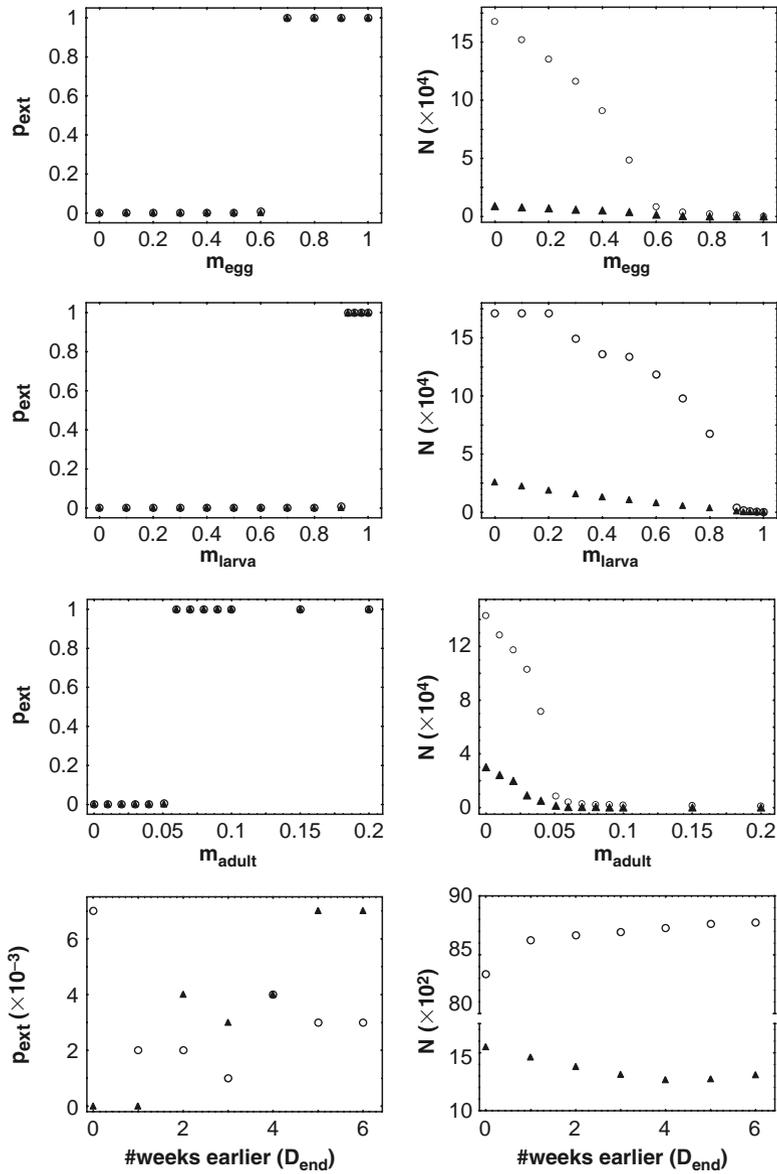
Changes in mortalities assumed for eggs, larvae and adults strongly affected extinction probabilities and the mean annual number of adults under both temperature scenarios. For all life stages and both temperature conditions studied, the extinction probability showed a logistic increase with increasing mortalities, whereas mean annual numbers of adults logistically decreased (Fig. 2, Table 2). Under temperature conditions as observed at Prappach, the strength in the shift in the termination of diapause towards the beginning of the year linearly decreased the extinction probabilities, whereas the mean annual population size hyperbolically increased for earlier terminations (Fig. 2, Table 2). In contrast, under conditions as observed at Hammelburg, an earlier termination of diapause had no effect on the extinction probability whereas size decreased in a quadratic manner (Fig. 2, Table 2). However, for both populations the latter effects on population size and extinction probability were very small.

## 4 Discussion

### 4.1 *Differing Population Dynamics at Prappach and Hammelburg*

Based on our simulation results, we were able to show that differences in temperature conditions and food limitation account for the differences in population development observed between Prappach and Hammelburg. This hypothesis had been proposed by Gottschalk et al. (2003) based on field observations and laboratory experiments. The trend observed in population development at Prappach was best explained by the model assuming weak food limitation ( $K_{\text{Prappach}} > 1,000$ , higher carrying capacities that implement food availability). Rather constant population sizes as observed at the warmer Hammelburg area were only obtained under strong food limitation ( $200 \leq K_{\text{Hammelburg}} \leq 500$ , small carrying capacities).

Population size at Prappach is mainly driven by annual temperature conditions, but density regulation by shortage of food is weak. The more unfavourable thermal conditions allow for lower reproduction than at Hammelburg (Köhler 1999; Waltert et al. 1999) and, thus, in colder years to a poorer compensation of reductions in population size (between 1996 and 1999) than at Hammelburg. Introducing a stronger



**Fig. 2** Results of the sensitivity analysis performed for life stage mortalities ( $m_{egg}$ ,  $m_{larva}$ ,  $m_{adult}$ ) and the assumed end of egg diapause. Extinction probabilities ( $p_{ext}$ ) and mean annual numbers of adults ( $N$ ) were estimated for temperature conditions as observed at Prappach (open dots) and Hammelburg (filled triangles). Capacities and initial population sizes assumed were 3,000 adults for Prappach and 30,000 adults for Hammelburg. Tested model parameter values are summarized in Table 1. Regression functions fitted are given in Table 2

**Table 2** Results of regression analysis performed for the sensitivity analysis of life stage mortalities ( $m_{egg}$ ,  $m_{larva}$ ,  $m_{adult}$ ) and the assumed end of egg diapause ( $D_{end}$ )

Site	Parameter	Estimate	Regression model	N	R <sup>2</sup>
Prappach	$m_{egg}$	$p_{ext}$	Logistic	11	0.999
	$m_{larva}$	$p_{ext}$	Logistic	14	0.999
	$m_{adult}$	$p_{ext}$	Logistic	13	0.999
	$D_{end}$	$p_{ext}$	Linear	7	0.938
Hammelburg	$m_{egg}$	$p_{ext}$	Logistic	11	0.999
	$m_{larva}$	$p_{ext}$	Logistic	14	0.806
	$m_{adult}$	$p_{ext}$	Logistic	13	0.999
	$D_{end}$	$p_{ext}$	Linear	7	n.s.
Prappach	$m_{egg}$	$\bar{N}$	Logistic	11	0.996
	$m_{larva}$	$\bar{N}$	Logistic	14	0.983
	$m_{adult}$	$\bar{N}$	Logistic	13	0.995
	$D_{end}$	$\bar{N}$	Hyperbolic	7	0.990
Hammelburg	$m_{egg}$	$\bar{N}$	Logistic	11	0.991
	$m_{larva}$	$\bar{N}$	Logistic	14	0.993
	$m_{adult}$	$\bar{N}$	Logistic	13	0.977
	$D_{end}$	$\bar{N}$	Quadratic	7	0.944

Regression functions applied for extinction probabilities ( $p_{ext}$ ): logistic:  $p_0(x) = \frac{1}{1 + \exp(-(a + bx))}$ , and linear:  $p_0(x) = ax + b$ ; for mean annual numbers  $\bar{N}(x) = \frac{c}{1 + \exp(-(a + b(1-x)))}$ , hyperbolic:  $\bar{N}(x) = \frac{(c-a)x}{b+x} + a$ , and quadratic:  $\bar{N}(x) = a(x-b)^2 + c$ ; where  $a, b, c$  denote parameters of fitted functions and  $x$  mortalities or ends of diapause, respectively. Tested parameter values are summarized in Table 1. Capacities and initial population sizes assumed were 3,000 adults for Hammelburg and 30,000 adults for Prappach

food limitation ( $K_{Prappach} < 1,000$ ) that increases overall mortalities of juveniles and adults resulted in frequent extinctions of the population due to the lower reproductive output of the population in this colder habitat. However, the population sizes in 1997, 1998 and 1999 were strongly overestimated by the model which might be attributed to increased mortalities of life stages in the natural population due to the cool and wet weather in these years (Richards and Waloff 1954; Ingrisch and Köhler 1998). Such raises in mortalities due to precipitations are not implemented in the model, but have a significant detrimental effect on population size as our third simulation study predicts (Fig. 2, Table 2). Furthermore, indirect effects of the weather such as changes in vegetation that reduce food availability also have a negative influence on population size, which is omitted in the model.

In contrast, population size at Hammelburg is strongly controlled by food. The favourable temperature conditions at this site allow in general for a higher reproduction rate than at Prappach (Waltert et al. 1999). While the reproductive output of the population at Prappach is mainly weather-driven (not limited by food because of high abundance of nutritious seeds, high carrying capacity), the reproductive output at Hammelburg is more constant. It does not strongly increase in warmer years due to low food abundance and does not intensively decrease during colder years due to the more favourable microclimate. The more even and higher production of offspring caused by warmer conditions at Hammelburg stabilizes population

dynamics, leads to more constant but lower annual population sizes and allows a faster recovery of population size even in unfavourable years (1997, 1998 and 1999). A reduction in food limitation (higher carrying capacities) increased mean annual population sizes, which were predicted for Hammelburg. In this situation, population development was driven by annual temperature conditions resulting in stronger annual fluctuations of population size. However, conversely to Prappach, our model underestimated population sizes at Hammelburg in the last 3 years. This underestimation is explainable by individual habitat choice of animals in heterogeneous habitats. *P. albopunctata*, like several other orthopteran species (Ehrlinger 1995; Kindvall 1996), prefers areas within habitats with more favourable microclimatic conditions if ambient environmental conditions are worse (Gottschalk 1996). This behaviour increases the individual reproductive output.

Although the rate of detecting density dependence is strongly affected by the length of the time series (Hassel et al. 1989; Woiwood and Hanski 1992; Holyoak 1993), various authors have successfully tested that fluctuations in insect population size can resemble density-independent random walks as observed at Prappach (e.g. Thomas et al. 1994; Whittaker and Tribe 1998) or density-dependent walks as found at Hammelburg (e.g. Alyokhin et al. 2005; Kölzsch et al. 2007). Even for orthopteran species, correlations between temperature and population densities have been reported in the literature (Kemp and Onsager 1986; Haes et al. 1990).

#### **4.2 *Extinction Risk of Our Two Natural Populations Under Current Thermal Conditions***

Based on our simulations, we can conclude that the survival of none of the two natural populations is secured. The minimum viable size was found for a carrying capacity of 3,000 individuals under temperature conditions as observed at Hammelburg (1,828 adults) and was 10-fold higher under conditions as observed at Prappach (8,341 adults, Griebeler and Gottschalk 2000a). However, the mean population size estimated between 1993 and 1999 was 1,390 adults at Prappach and 614 adults at Hammelburg (Gottschalk et al. 2003), which are both below these limits. Since both study areas are surrounded by a number of additional local populations (Griebeler and Gottschalk 2000b; Gottschalk 1996) and the species is able to capture large distances (maximal observed distance is 2–3 km (Walter and Gottschalk 1998)), the protection of these complexes as metapopulations might increase species survival at both study areas. An estimation of the total population size at the Prappach region (6,835 adults) that was calculated by Griebeler and Gottschalk (2000b) is still below the mean annual number of adults derived for a carrying capacity of 30,000 adults (8,341 adults). This suggests that additional conservation measures (grazing or mowing) are needed in this region to enable species persistence. Grazing and mowing improve temperature conditions and cause a shortage of food which stabilizes population dynamics and results in smaller, but safer populations as observed for Hammelburg.

### 4.3 Survival of *P. albopunctata* Under Climate Change

Our simulations demonstrate that thermal conditions in the habitat have a severe impact on the extinction risk of the grey bush cricket. The decreasing extinction risk with higher temperatures is caused by the higher reproductive output of adults under improved temperature conditions (Köhler 1999; Waltert et al. 1999). Based on this observation, one might conclude that *P. albopunctata* will be able to successfully survive global environmental warming. However, increasing mortalities, which are realistic for increasing precipitation (Richards and Waloff 1954; Ingrisch and Köhler 1998), will highly influence the population sizes and extinction probabilities as predicted by our model (Fig. 2, Table 2). A slight increase in mortalities assumed for eggs, larvae and adults leads to a rapid extinction of the population under both temperature conditions studied (Fig. 2, Table 2).

How far biotic conditions for the bush cricket will change in the future is unpredictable, as they range from intra-specific interactions (competition for food) to multi-species interactions (e.g. inter-specific competition, predation). Based on our model, we suggest that if mortalities caused either by abiotic or biotic factors will decrease, this will favour survival of *P. albopunctata* under increasing temperatures whereas an increase in mortalities is detrimental for survival. However, *P. albopunctata* expanded the inhabited area in the Prappach region by colonizing meadows with quite dense vegetation (E. Gottschalk, pers. observation) during warm years. An increase in the potential habitat area under a warmer climate could be predicted based on this observation.

Several studies have demonstrated changes in temporal patterns of species caused by increasing temperatures (Sparks and Carey 1995; Roy and Sparks 2000). Since *P. albopunctata* eggs do not need frost as stimulus for hatching, and larvae hatch earlier at higher temperatures (Ingrisch 1985), increased spring temperatures may shift egg hatching towards the beginning of the year. Our simulation results show that this shift in the phenology has a negligible small effect on both the extinction probability and the size of the two populations if life stage mortalities are not altered. The adult mortality observed at both study areas which we assumed in our model (5.1%) results in about 20 days for mean adult life time. This limitation inhibits that the population expanded its phenology towards the warmer autumn in our simulation, but whether an elongation is possible ultimately depends on future intra- and inter-specific interactions influencing adult mortality.

Future temperatures as well as availability and distribution of rainfall will also affect food availability for the species. Increasing precipitation and temperatures affect vegetation and might reduce food availability. A shortage of food (decreasing  $K$ ) will increase the risk of population extinction independent of temperature conditions assumed as predicted by our model. Whether a detrimental effect of precipitation and temperature on the availability of food might be compensated by the positive effect of increasing temperatures on reproduction needs further empirical and theoretical studies.

## 5 Conclusions

This study shows that simulation models are able to support conservation biologists in their understanding of processes that affect population extinction under current and future habitat conditions, and that models can also help to develop conservation measurements. Our example of different population dynamics generated by differences in temperature conditions and food availability (vegetation cover) demonstrates a general problem of obtaining estimates for MVPs for any temperature-dependent species. The MVP is not a pure species-specific value; it also severely depends on habitat conditions, which influence population dynamics. Forecasting species survival based on data of any population without adapting the specific local conditions will greatly impair prognosis and might lead to accidental extinction of the species.

Relict species, however, may react more pronouncedly to changes in habitat conditions caused by anthropogenic land use or climate change than our model species *P. albopunctata*. While relict species are also often restricted to patchy habitats, their habitats are scarcer than those of the grey bush cricket and they are often more specialized than the cricket. If the habitats of relict species are altered or destroyed due to global change, populations will be much more endangered than those of any other species that has a broader range distribution, shows a higher ecological and possibly genetic plasticity and may find other suitable habitats by dispersing.

**Acknowledgements** We are grateful to two anonymous reviewers and Dana Berens who provided helpful comments on the manuscript.

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# Is the ‘Lost World’ Lost? High Endemism of Amphibians and Reptiles on South American Tepuís in a Changing Climate

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**Abstract** The Pantepuí region of the Guyana Shield of Northern South America harbors a unique biota. It is characterized by high local species endemism, especially applicable to its table mountain-like outcrops, the tepuís. For this region, also known as the ‘Lost World’, different hypotheses have been proposed to explain its Biogeography. We suggest that the amphibian and reptile communities’ suit well as target group to study the hypotheses since these vertebrates are relatively well documented through comprehensive species lists and are less mobile than other terrestrial vertebrates. However, records for the majority of endemic amphibian and reptile species naturally numbered only a few. We hence used extant dimensions of the vegetation zones associated to amphibians and reptiles. By running maximum entropy-based Climate Envelope Distribution Models (CEDM), these were projected to climatic scenarios as suggested for the Last Glacial Maximum (LGM, 21,000 years BP). Our results promote the Cool Climate hypothesis for diversity and distribution of amphibians and reptile communities in the Pantepuí region, in which species’ geographic ranges were displaced followed by speciation along with the climate change during glacial-interglacial Quaternary cycles. Moreover, we studied the impact of expected future anthropogenic global warming on these vegetation zones. Climate change scenarios used, consider demographic, politico-economic, social and technological futures at different degrees with either more or less environmentally conscious, regionalized solutions to economic, social and environmental sustainability. For the ‘Lost World’, our CEDM results demonstrated

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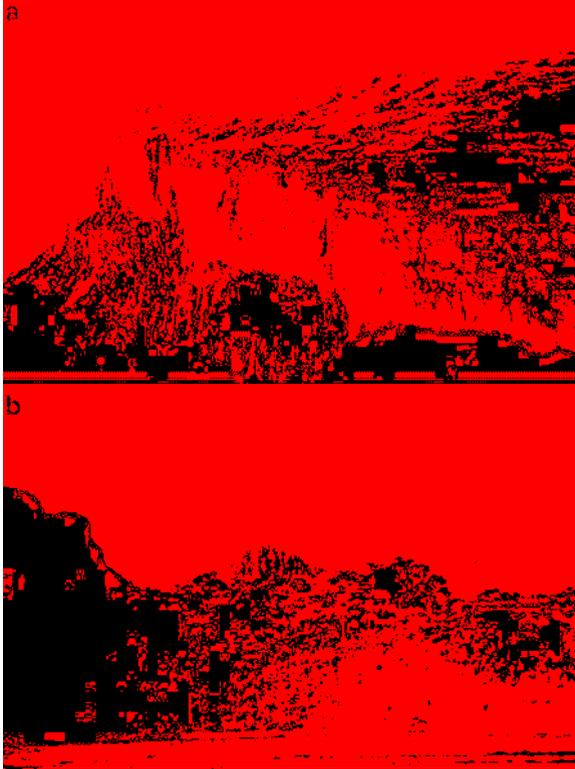
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a strong impact of climate change to vegetation zones resulting in a considerable decrease during future anthropogenic global warming in terms of habitat suitability. For amphibians and reptiles this may mean habitat loss and decline or extinction. However, nothing is known about specific climatic niche breadths (which may be great due to survival under climatic conditions with considerable daily change) or the possibility to adapt over generations (which may be unlikely due to the rapid warming expected) of Pantepuí amphibians and reptiles. Further studies and monitoring efforts are necessary towards a better understanding of the problem and to develop conservation action plans.

## 1 Towards an Understanding of the Biogeography of the ‘Lost World’

One of the most famous fantasy adventure novels has been Sir Arthur Conan Doyle’s 1912 ‘The Lost World’. Purely science fiction, the geological formation of this story finds its paradigm in the real world – the Precambrian Guyana Highlands in South America. This region, which is also known as the Pantepuí harbors an extraordinarily high biodiversity. Pantepuí is situated on the Precambrian Guyana Shield, which had been separated from the African Shield by a continental drift around 80–100 Myr ago (Edmond et al. 1995). Endemism rates are especially remarkable in both plants and animals and at different taxonomic levels (Berry et al. 1995). For examples, in amphibians and reptiles, two well surveyed vertebrate groups in this region, 77.3% (i.e. 75 of 97) and 54.8% (i.e. 34 of 62), respectively, of the known species are endemics (McDiarmid and Donnelly 2005). One reason for this is the unique geomorphology of this region, characterized by isolated table mountains, the tepuís (Fig. 1.). These are remnants of ancient erosion surfaces that have been isolated during the Gondwana breakup, accompanied by the formation of major river systems (Briceno and Schubert 1990; Huber 1994; Edmond et al. 1995). Today, the Pantepuí region constitutes a singular biogeographic province of about 5,000 km<sup>2</sup>, which is split into numerous topographical islands, each about 1,500–3,000 m above sea level (Fig. 2; Huber 1994).

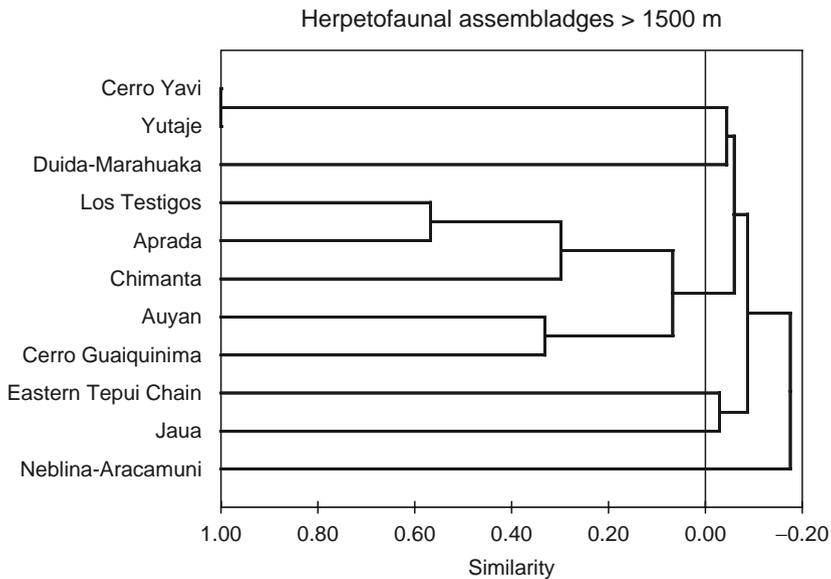
Different aspects of the Pantepuí region have been of particular interest to various writers (e.g. Hoogmoed 1979; Duellman 1999; Gorzula and Señaris 1998; McDiarmid and Donnelly 2005; Rull 2005a,b; Señaris and MacCulloch 2005; MacCulloch et al. 2007; Rull and Nogué 2007) and Pantepuí region has been suggested to be well suitable to study biogeographic and evolutionary theories (Rull 2005a,b, 2007). In a recent synopsis, McDiarmid and Donnelly (2005) summarized the different hypotheses trying to explain diversity patterns within the region. The Distance Dispersal hypothesis (1) was derived from observations on the Pantepuí bird fauna by Mayr and Phelps (1967). These authors proposed that some Andean bird species reached the tepuís crossing the valleys of the Orinoco and Negro Rivers and subsequently became established at some of the Western tepuís. Further dispersal through ‘island hopping’ across the Pantepuí to the Eastern massifs is suggested



**Fig. 1** Impressions from the 'Lost World' or Pantepuí region of South America: Cerro Guaiquinima (a) and El Venado Tepuí (b). Note the strict distinctness in vegetation between basement and summit of these table mountains, here referred to as 'upland' and 'tepuí', respectively. Photos: A. Schlüter

to have taken place, followed by speciation. The Distance Dispersal hypothesis assumes a high mobility of organisms and is therefore only applicable to few organisms. The Habitat Shift hypothesis (2) was introduced by Mayr and Phelps (1967) to explain for more than 36% of the Pantepuí avifauna, and Hoogmoed (1979) further suggested that it may also be applicable to the majority of amphibians and reptiles living at higher elevations. Basically, the Habitat Shift hypothesis assumes that elements of the Pantepuí fauna derived from lowland ancestors that adapted to intermediate and highland habitats and subsequently formed distinct species. Maguire (1970) and Brewer-Carías (1978) suggested that biodiversity, especially the high degree of endemism, in the Guyana Highlands is the result of a long history of evolution in isolation over several geological eras: Lost World hypothesis (3). In a contrasting view, alternating up- and downward displacement during glacial-interglacial Quaternary cycles has been proposed (e.g. Chapman 1931; Tate 1939; Mayr and Phelps 1967; Steyermark and Dunsterville 1980; Rull 2004a,b, 2005a,b). According to this Cool Climate hypothesis (4), the main diversification





**Fig. 3** Comparison of herpetofaunal assemblages at massifs in the Pantepuí region. Most herpetofaunas show limited overlap only, even when compared to neighbouring massifs. Cluster analysis was performed based on a species list of amphibians and reptiles compiled from Donnelly and Myers (1991), Mägdefrau et al. (1991), McDiarmid and Donnelly (2005), MacCulloch et al. (2007), Means and Savage (2007), Schlüter and Rödder (2007), Myers and Donnelly (2008), Rödder and Jungfer (2008)

assemblages as a result of the high degree of local endemism becomes obvious (Fig. 3.). The wide lack of overlapping faunal elements makes broad scale assessments and evaluation of hypotheses difficult. Phylogeographic analyses would be the most suitable tool for an evaluation of the hypotheses formulated above; however, DNA samples, as required, are largely unavailable.

GIS-based Climate Envelope Distribution Models (CEDMs) may provide an easy-to-use-alternative. In recent times, there have been several examples using CEDMs for species or habitat climate envelopes under past, present and future climate scenarios (e.g. Hugall et al. 2002; Bonaccorso et al. 2006; Peterson and Nyári 2008). Such approaches rely on the assumption that climatic tolerances of species are the primary determinants of their current distributions and that specific climatic niches are conservative, at least within an evolutionary short time frames of some hundreds to thousands years (e.g. Wiens and Graham 2005; but see Pearman et al. 2007).

Peterson and Nyári (2008) and Carnaval and Moritz (2008) suggested that spatial modelling of species or habitats under current and palaeoclimate regimes can generate hypotheses about the potential existence and the extent of stable (refugial) regions. On the other hand spatial models may likewise provide information on past – or future – extents of current refuges.

### 3 Principles of CEDMs

Prior to CEDM performance, it is necessary to compile a list of species records (i.e. geographic coordinates) and of values of climatic variables which are suitable to describe the actual range of the target species. Fortunately, GIS-maps of environmental variables help to find suitable data sets in many regions (e.g. <http://www.worldclim.org>). A subsequent step involves the development of a multidimensional view of the climate envelope of a species, which is a challenge given the complex nature of a species' ecological niche (Peterson and Vargas 1993). In simple words, in CEDMs as mentioned here, climatic information for the presence of species localities are summarized to an 'ideal' climatic niche for the target species which is afterwards compared to climatic conditions at localities of query, i.e. from which the presence or absence of the target species is unknown. The results are geographic maps showing different degrees of similarity (interpreted as suitability) of a region with the 'ideal' climatic niche. The selection of a moderate algorithm for the computation of a CEDM depends on the amount of distribution records available and their quality.

One of the earlier applied algorithms for the presence of species data is BIOCLIM (Nix 1986; Busby 1991), as implemented in DIVA-GIS 5.4 (Hijmans et al. 2002; <http://www.diva-gis.org>). It develops CEDMs by intersecting the ranges inhabited by the species along each environmental axis. More sophisticated algorithms are GARP (Stockwell and Noble 1992; Stockwell and Peters 1999), DOMAIN (Carpenter et al. 1993), Maxent (Phillips et al. 2006) and LIVES (Li and Hilbert 2008). The machine-learning Maxent (see below) is often superior to most other methods and hence becomes more and more distributed (e.g. Elith et al. 2006). If records of apparent species absence are available for modeling other algorithms, such as 'artificial neuronal networks', 'classification and regression trees', 'generalized additive models' or 'generalized dissimilarity models' can be applied. These algorithms for example are implemented in the BIOMOD tool (Thuiller 2003).

Unfortunately, the high degree of endemism in the Pantepuí region causes difficulties when using CEDMs at the species level, since a minimum amount of 10–20 distribution records pending on the algorithm applied is at least necessary (e.g. Elith et al. 2006). Looking at the herpetofaunal assemblages, 109 out of 159 (i.e. 97 amphibian and 62 reptile) species are only known from a single tepuí (McDiarmid and Donnelly 2005). As a result, even in this relatively well documented group in the region, for most species CEDMs cannot be seriously generated. Since species distributions are commonly related to specific habitats, one possibility is to perform CEDMs for habitats rather than for a single species using a random set of distribution points within a target habitat. Such an approach was well performed by Carnaval and Moritz (2008) for the identification of climatically stable refugia during the Last Glacial Maximum (LGM), 21,000 years BP, in the Brazilian Atlantic forest.

## 4 Reconstructing Connectivity Patterns Within Pantepuí Under LGM Climate

For CEDM calculation, MaxEnt 3.2.1 (Phillips et al. 2006; <http://www.cs.princeton.edu/~shapire/maxent>) was used to assess potential distributions of vegetation zones during LGM. Maxent is a machine-learning algorithm following the principles of maximum entropy (Jaynes 1957). In order to study responses in terms of potential distributions of vegetation zones in the Guyana Highlands, especially the tepuís under different climate scenarios, distribution data points are necessary for CEDM calculation. For this, we generated 10,000 randomly distributed points spanning from latitude 0.3° S to 8.7° N and longitude 68.7° W to 57.6° W with DIVA-GIS 5.4 (Hijmans et al. 2002; <http://www.diva-gis.org>). This spatial coverage was deliberately made larger than those occupied by the Pantepuí region to ensure that training points later used to generate CEDMs under current-day climate encompassed the full extension of the climate landscape. Altitudinal properties at each of the 10,000 points were extracted and divided into two altitudinal range classes, termed 'upland' and 'tepuí' (see McDiarmid and Donnelly 2005; MacCulloch et al. 2007): i.e. 804 points at 800–1,500 m above sea level and 65 points at  $\geq 1,500$  m above sea level. For a better understanding of the discrimination between these two terms see Fig. 1b.

Information on current climate was obtained from the WorldClim database, version 1.4, which is based on weather conditions recorded between 1950 and 2000 with grid cell resolution 2.5 min (Hijmans et al. 2005; <http://www.worldclim.org>). It was created by interpolation using a thin-plate smoothing spline of observed climate at weather stations, with latitude, longitude and elevation as independent variables (Hutchinson 1995, 2004).

For paleoclimate, General Circulation Model (GCM) simulations from the Community Climate System Model (CCSM) assuming a temperature decrease of approximately 4 °C for the target region were provided by R. J. Hijmans (<http://www.ccsm.ucar.edu/>; Kiehl and Gent 2004). Original GCM data were downloaded from the PIMP2 website (<http://www.pimp2.cnrs-gif.fr/>) with spatial resolution of roughly 300×300 km. Surfaces were created at 0.04° spatial resolution via the following procedure: first, the difference between the GCM output for LGM and current-day conditions was calculated. These were then interpolated to the 0.04° resolution grid using the spline function in ESRI ArcInfo with the tension option. Eventually, the interpolated difference was added to the high-resolution current-day climate data set from WorldClim and LGM bioclimatic coverage using DIVA-GIS. This procedure has the dual advantages of producing data at a resolution relevant to the spatial scale of analysis and of calibrating the simulated climate change data to the actual observed climate data.

The layers for each climate scenario included the minimum and maximum temperatures and the mean precipitation per month (= 36 climatic parameters) derived from the WorldClim 1.4 interpolation model. Based on these, seven bioclimatic

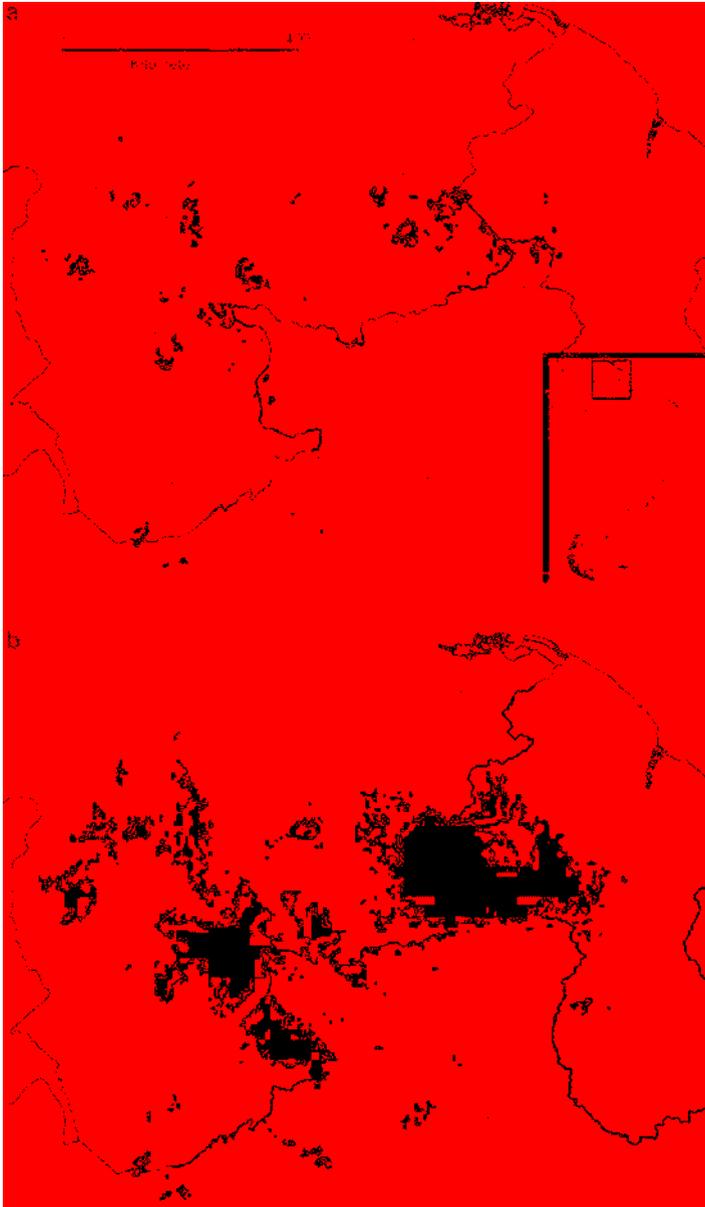
parameters for each climate scenario were generated with DIVA-GIS: ‘annual mean temperature’, ‘temperature seasonality’ (standard deviation of monthly mean temperature  $\times 100$ ), ‘mean temperature of the warmest quarter’, ‘mean temperature of the coldest quarter’, ‘annual precipitation’, ‘precipitation of the wettest quarter’ and ‘precipitation of the driest quarter’. These parameters reflect the availability and range of thermal energy and humidity and are suitable for CEDM projections between different climate scenarios (e.g. Carnaval and Moritz 2008). Bioclimatic parameters were exported from DIVA-GIS as \*.grd/\*.gri files.

CEDMs were calculated for both altitudinal range classes separately and results are shown in Fig. 4. Maxent allows for model testing by calculation of the Area Under the Curve (AUC), referring to the ROC (Receiver Operation Characteristic) curve using e.g. 25% of the records as test points and the remaining ones for training (Hanley and McNeil 1982; Phillips et al. 2006). We acknowledge that currently there has been some doubt raised on the reliability of AUC (Lobo et al. 2008). But for a lack of a better method and because AUC has been recommended and applied for ecological applications by other authors (e.g. Pearce and Ferrier 2000; Elith et al. 2006; Phillips et al. 2006), we hereby continue using it.

AUC values range from 0.5 (i.e. random) for models with no predictive ability to 1.0 for models giving perfect predictions. According to the classification of Swets (1988), AUC values  $>0.9$  describe ‘very good’,  $>0.8$  ‘good’ and  $>0.7$  ‘useful’ discrimination ability. We received ‘very good’ AUC values in the models for ‘upland’ ( $AUC_{\text{training}}=0.972$ ;  $AUC_{\text{test}}=0.969$ ) and ‘tepuí’ ( $AUC_{\text{training}}=0.998$ ;  $AUC_{\text{test}}=0.998$ ).

Visual comparisons of our CEDM for current conditions and elevation, within the study region confirmed high overlap (Figs. 2 and 4a). CEDMs of ‘upland’ and ‘tepuí’ under LGM conditions revealed that the respective areas were apparently much larger supporting the Cool Climate hypothesis of Rull (2005a). Temporal connections between the ‘tepuís’ during glacial and interglacial oscillations were present and may have allowed exchange of biota. According to our models, at least, the whole upland and the Eastern massifs, such as the Ayuan, Los Testigos, Chimanta, Aprada the Eastern Tepuí Chain the Cerro Yavi and Yutaje massifs (Fig. 2) were connected, whereby the remaining ones exhibited no or limited contact (Fig. 4b). These findings largely coincide with those of Rull and Nogué (2007), who performed a more simple assessment of connectivity patterns assuming an adiabatic temperature gradient and cooling of about  $4.1^{\circ}\text{C}$ .

A comparison of similarities between herpetofaunal assemblages inhabiting different massifs (Fig. 3.) showed that faunas at Cerro Yavi and Yutaje are mostly alike, followed by a clade summarizing Eastern massifs (Los Testigos, Aprada, Chimanta, Ayuan and Cerro Guiaquinima). Duida-Marahuaka, Jaua, Neblina-Aracamuni and the Eastern Tepuí Chain are inhabited by more different assemblages. Therefore, we conclude that the connectivity patterns suggested by our palaeoclimate CEDM and hence the Cool Climate hypothesis may explain most of the similarity among herpetofaunal assemblages observed herein, but not entirely. The relatively high similarity between Cerro Guiaquinima and the other Eastern massifs (e.g. Ayuan massif) cannot be well explained with our CEDM results showing



**Fig. 4** Potential distributions of 'upland' (*grey*) and 'tepuí' climate envelopes (*black*) under current (**a**) and Last Glacial Maximum (CCSM) conditions, 21,000 years BP (**b**)

no connection between them. Also, the distinctness of the herpetofauna of the Eastern Tepuí Chain, which may have been connected to the other Eastern massifs under LGM conditions, is remarkable.

McDiarmid and Donnelly (2005) argued that the high level of endemic amphibian and reptile species may cast some doubt on the Cool Climate hypothesis. Our results confirm that faunal exchange between most ‘tepuís’ was restricted, but possible during the LGM within the Eastern massifs at least. This geographic pattern is also reflected when comparing herpetofaunal assemblages, although the high degree of endemism causes remarkable differences even between geographically close massifs (Fig. 3), e.g. the Ayuan, Chimanta and Los Testigos massifs located within a distance of less than 100 km (Fig. 2).

We expect that the extent of faunal exchange during glacial periods depended on the establishment abilities of the invaders in existing faunas. Myers and Donnelly (2001) found that some endemic species have phenotypically and ecologically sibling counterparts on other tepuís. MacCulloch et al. (2007) made similar observations regarding Mt. Roraima and adjacent tepuís. This appears to be especially applicable to frogs in the genera *Anomaloglossus*, *Oreophrynella* and *Pristimantis* as well as in snakes in the genus *Thamnodynastes*. These species were suggested to comprise monophyletic highland groups rather than separate invasions from lowlands (Myers and Donnelly 2001) and, considering the geological age of the Pantepuí region allowing speciation, many of them may represent sister species. These taxa occupy similar ecological niches (Myers and Donnelly 2001) which would hamper the establishment of one species at tepuís inhabited by a similar species, so much so even if ‘tepuís’, were connected during the glacial periods, faunal exchange may have been limited, explaining – at least in parts – the distinctness of ‘tepuí’ herpetofaunas.

## 5 Assessing Possible Impacts of Future Anthropogenic Climate Change

Next to the interesting opportunity to study relevant biogeographic and evolutionary aspects related to past climate, the unique amount of endemic taxa of Pantepuí may be of certain interest when watching through a window into the future. There is little doubt that within a few decades, the worldwide climate will become warmer due to human impact (IPCC 2007).

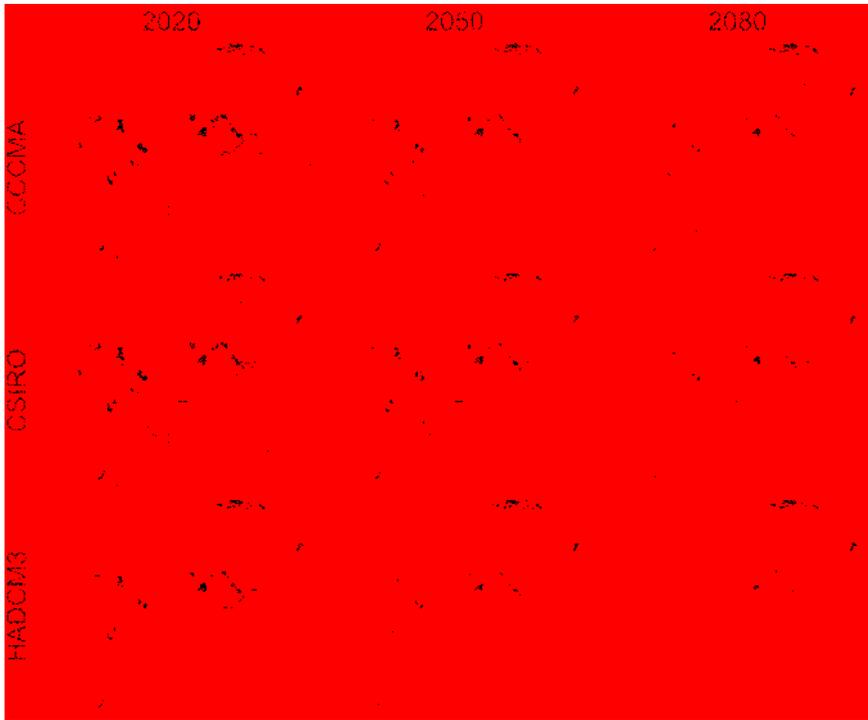
Efforts in global biodiversity conservation have underlined the relevance of Pantepuí as a region which deserves protection, as it is listed as one of the WWF/IUCN Neotropical Plant Diversity centres (Pantepuí or SA-2; WWF and IUCN 1997). That is why more than 70 % of the Guyana Highlands is under official protection (Berry et al. 1995). However, as one of the dilemmas of current conservation strategies, land protection alone is not operable to threats caused through future anthropogenic global warming (Rull and Vegas-Vilarrúbia 2006; Rull 2007). Rull and Vegas-Vilarrúbia (2006) tentatively estimated that one tenth to one third of suitable habitat will be subject to loss when temperature increases for 2–4°C over the forthcoming decades. As outlined above, CEDMs provide a powerful tool

towards quantification and the response of species to – past and future – climate change in geographic space in terms of habitat suitability.

In order to quantify the expected impact of future anthropogenic global warming to vegetation zones defined above, we computed CDEMs in the manner as described above. For this purpose, we used climate change projections based on the CCCMA, CSIRO and HADCM3 (Flato et al. 2000; Gordon et al. 2000) models and the emission scenarios reported in the Special Report on Emissions Scenarios (SRES) by the Intergovernmental Panel on Climate Change, IPCC (<http://www.grida.no/climate/ipcc/emission/>). A set of different families of emission scenarios was formulated based on future production of greenhouse gases and aerosol precursor emissions. The SRES scenarios of A2a and B2a were used in this study. Each scenario described one possible demographic, politico-economic, social and technological future. Scenario B2a emphasizes more environmentally conscious, more regionalized solutions to economic, social and environmental sustainability. Compared to B2a, scenario A2a also emphasizes regionalized solutions to economic and social development, but it is less environmentally conscious. For quantitative comparisons between different projections we counted the number of grid cells classified as suitable per vegetation zone with DIVA-GIS.

Our models revealed that under global warming, potential distributions of both 'upland' and 'tepuí' climate envelopes became drastically smaller (Figs. 5 and 6). Applying scenario A2a resulted in a stronger decrease of suitable habitat in both 'upland' and 'tepuí' than scenario B2a. In general, the results of scenarios derived from CCCMA and CSIRO models were roughly similar, suggesting less reduction of potentially suitable habitat than scenarios derived from the HADCM3 model (Table 1). Our findings are coincident with those of Rull and Vegas-Vilarrúbia (2006) who, in a preliminary study which was not subject to CEDM, indicated that 'upland' and 'tepuí' climate envelopes will remarkably shrink under anthropogenic global warming. Different to other Neotropical mountain ecosystems (e.g. Bush 1994), physical circumstances will allow for almost no vertical migration. Now, the question is how will the Pantepuí amphibian and reptile endemics respond to this? Decline and at worst extinction – or is there a chance of survival?

Although it is widely accepted that species' climate envelopes behave conservative, they may have the ability to adapt to climate change over short time periods so that they can compensate potential distribution range loss (e.g. Peterson et al. 1999; Wiens and Graham 2005). This depends on the succession of generations driven by natural selection, ecological potency (i.e. niche breadth) and change rates of the environment and species (e.g. Holt and Gomulkiewicz 2004; Thomas et al. 2004; Pearman et al. 2007). However, it is actually unknown how large the climatic niche breadth of Pantepuí amphibian and reptile (or any other) species is. Also, their adaptability over generations remains hidden. But taking the suggested rapid warming, it may be doubted that vertebrates, even with comparatively short generation succession as amphibians and reptiles, are able to succeed. Today, the Guiana Highlands exhibit a mild perhumid climate with mean annual temperature 12–18°C and total annual rainfall 2,500–3,000 mm (Berry et al. 1995), while for the next

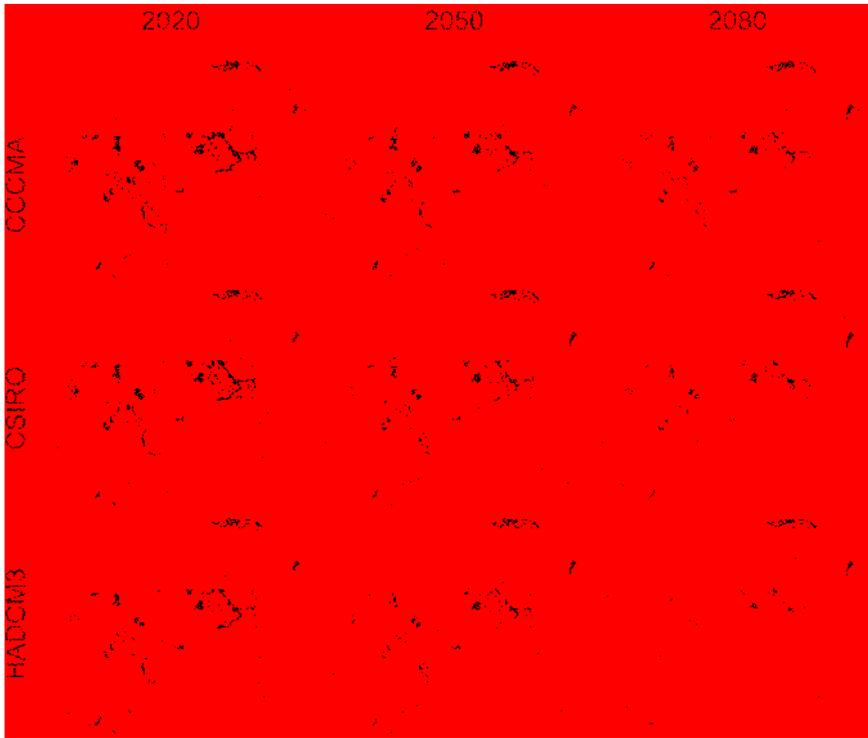


**Fig. 5** Potential distributions of ‘upland’ (grey) and ‘tepuí’ climate envelopes (black) under A2 conditions in 2020, 2050, and 2080, according to CCCMA, CSIRO and HADCM3 future climate change (i.e. anthropogenic global warming) scenarios

century there has been predicted a temperature increase of 1.8–5.1°C (IPCC 2007). In contrast, during the last postglacial warming, it has been shown that Guyana Highlands plant species were able to ‘handle’ a temperature increase of 0.025°C per century (Rull and Vegas-Vilarrúbia 2006) what is a 72–204 times lower rate than the IPCC prediction.

Regarding niche breath, the climate at the tepuí summits is characterized by weak variation niche between annual minimum and maximum temperatures, but high temperature ranges between day and night (e.g. up to 21°C within 6 h recorded at Chimantá; McDiarmid and Gorzula 1989). This daily temperature range indicates that especially tepuí species are perhaps unaffected by annual climate change even within few generations.

In order to have a better understanding of threats caused by climate change and of adaptation abilities, expected changes in vegetation cover in the Guyana Highlands need to be monitored and quantified spatially over the next decades.



**Fig. 6** Potential distributions of 'upland' (grey) and 'tepuif' climate envelopes (black) under B2a conditions in 2020, 2050, and 2080, according to CCCMA, CSIRO and HADCM3 future climate change (i.e. anthropogenic global warming) scenarios

**Table 1** Extension of potential distributions of climate envelopes of 'upland' (800–1,500 m elevation) and 'tepuif' (>1,500 m elevation) relative to current extents [%] in CEDMs for the years 2020, 2050 and 2080

Future climate change model	'Upland'			Future climate change model	'Tepuif'		
	2020	2050	2080		2020	2050	2080
<i>A2a</i>				<i>A2a</i>			
HADCM3	54.2	17.7	3.6	HADCM3	27.9	10.4	2.4
CSIRO	63.7	39.3	15.7	CSIRO	49.4	26.3	12.3
CCCMA	67.1	40.3	16.4	CCCMA	51.6	25.5	12.1
<i>B2a</i>				<i>B2a</i>			
HADCM3	54.1	32.8	8.9	HADCM3	30.6	15.4	5.6
CSIRO	61.0	39.8	24.6	CSIRO	47.9	26.6	17.5
CCCMA	68.4	48.8	34.0	CCCMA	52.4	30.9	22.0

**Acknowledgements** The work of DR was funded by the ‘Graduiertenförderung des Landes Nordrhein-Westfalen.’

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# Population Genetics and Ecological Niche Modelling Reveal High Fragmentation and Potential Future Extinction of the Endangered Relict Butterfly *Lycaena helle*

Jan C. Habel, Bettina Augenstein, Marc Meyer, Gabriel Nève, Dennis Rödder, and Thorsten Assmann

**Abstract** During the post-glacial warming, cold-adapted species shifted their distribution to higher latitudes and altitudes and became widely extinct over the European lowlands. The butterfly *Lycaena helle* shows this feature, and is currently distributed in highly isolated habitat remnants restricted to higher elevations over Central Europe. We analysed five polymorphic microsatellite loci and applied Climate Envelope Modelling. We detected strong genetic differentiation coinciding with the orographic structures of seven distinct mountain regions. This picture become underlined by deflecting levels of genetic diversity and the presence of private alleles, endemic for each single mountain area. Furthermore, genetic differentiation among populations within these mountain groups were detectable and reveal interrupted geneflow on a regional level. This genetic picture of a fragmented distribution coincides with the obtained pattern of potential suitable habitats given by a Climate Envelope Model. A scenario of further climate warming predicts a loss of the major parts of these areas and rising fragmentation of the remainings. The predicted extinction of some populations will cause the loss of unique alleles, which are recently restricted to the given populations.

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## 1 Introduction

Flora and fauna are affected by both historical and recent climatic oscillations (Hewitt 2001; Schmitt 2007). Most species respond to these climatic modifications through changes in their distribution patterns (Hewitt 1996). The nature of a species' reaction depends on its biotic and abiotic requirements: Thermophilic species become extinct over major parts of Europe during the last glacial period, but survived in Southern refugia (Habel et al. 2005). In contrast, continental species mostly survived in extra-Mediterranean and Eastern refugia and expanded their distribution range westwards (Schmitt 2007), during the postglacial, and cold-adapted species migrated to higher latitudes and altitudes, following the cold climate (Huntley and Webb 1989; Peterson et al. 1999; Parmesan and Yohe 2003; Root et al. 2003; Pearman et al. 2008; Varga and Schmitt 2008). In contrast to the situation in the Alps, where most species can still move and escape uphill, many of the species occurring in the Middle mountains of Europe have reached the altitudinal limit of these mountain areas and cannot compensate further climate warming by further uphill shifts. This means that the few remaining potential suitable habitat areas are highly fragmented, a situation which is aggravated by severe land-use changes. Living in isolation enhances processes of population dynamics (such as population fluctuations, Lesica and Allendorf 1995) and population stochasticity (Melbourne and Hastings 2008), resulting in the loss of genetic diversity in local sites through drift, which cannot be compensated by immigration from neighbouring populations (Hanski 1999). Such genetically impoverished populations often suffer from decreased fitness and viability due to inbreeding, which causes the accumulation of weakly deleterious alleles (Reed and Frankham 2003; Allendorf and Luikart 2006). The effects of this development have been analysed in theoretical and experimental studies (Frankham et al. 2002).

To investigate the effect of post-glacial and anthropogenic habitat fragmentation of boreo-montane species, we chose the endangered Violet Copper butterfly *Lycaena helle*. This species is adapted to cold and moist habitats (Bachelard and Descimon 1999; Steiner et al. 2006) complemented by specific habitat characteristics such as high abundance of its larval food plant *Polygonum bistorta* and habitat structures (shrubs and trees) (Turlure 2006). During the climatic warming of the postglacial period, the species escaped to higher altitudes and northwards (Habel et al. 2009a); today, most of the Central European populations are restricted to higher elevations and thus highly fragmented.

Genetic samples of butterfly specimens were collected on seven Western mountain areas (Pyrenees, Massif Central, Jura, Vosges, Madeleine mountains, Ardennes-Eifel and the Westerwald) to assess the genetic structure and effects of isolation. A Climate Envelope Model (CEM) (Phillips et al. 2006) was performed in order to identify and quantify climatically suitable habitats and possible connections between them (for further details see Rödder et al., 2009). Projection of the climatic envelope into the geographic space allowed us to assess the potential distribution of the butterfly under the current climate and a future climate change scenario

(cf. Araujo and Whittaker 2005). The combination of molecular analysis and niche modelling provided evidence of the species' distribution situation and the genetic response of highly fragmented suitable habitats. Both methods revealed an *extinction process* at habitat and species level.

## 2 Material and Methods

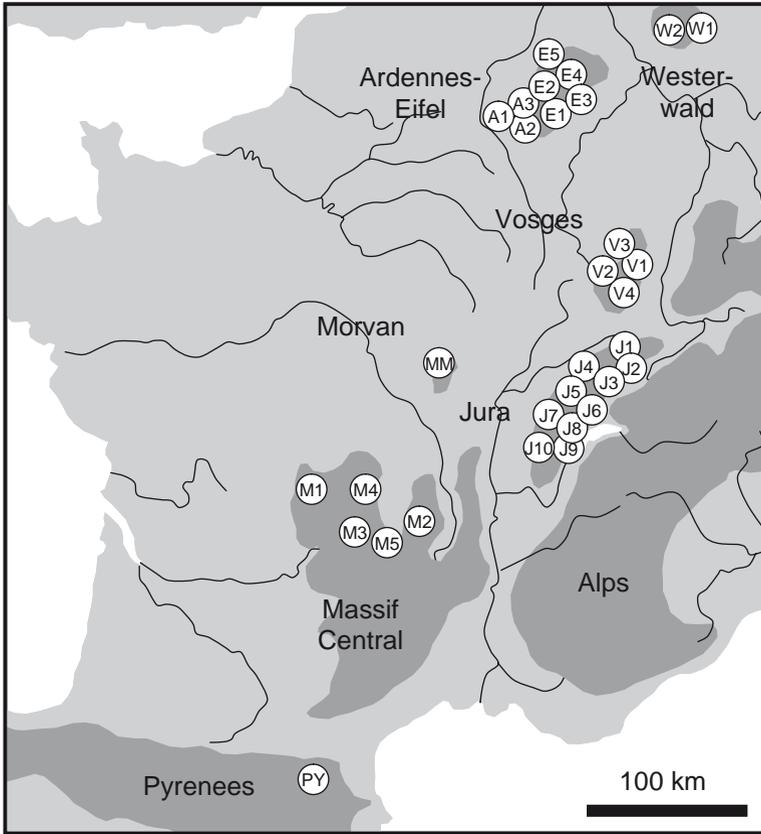
### 2.1 Study Species

The Violet Copper butterfly, *Lycaena helle* (Denis and Schiffermüller, 1775) is a boreo-montane species of the Palaearctic distributed over Central Europe, Fennoscandia, throughout Russia to the Amur region, China and Mongolia (Bozano 2001), and is present in cool and moist meadows. Essential habitat requirements are the food-plant *Polygonum bistorta* (in Europe), typical vegetation structures (Goffart et al. 2009) and specific moist and cold climatic conditions (Bachelard and Descimon 1999; Fischer et al. 1999; Turlure 2006). The species is classified as a generally sedentary butterfly (Bink 1992), but occasional movements among neighbouring sites or even rare long-distance movements have also been recorded (Bachelard and Descimon 1999). The recent situation which has involved habitat succession, industrialisation of land-use and afforestation has led to severe habitat fragmentation of this species, with severe genetic impact (Finger et al. 2009). The species is classified as one of the most endangered butterflies of Europe, listed in the European Red Data Book (Van Swaay and Warren 1999) and Appendices II and IV of the Habitat Directive (EEC, 92/43/EWG) of the European Union (Drews and Pretschner 2003).

### 2.2 Genetic Analysis

A total of 560 individuals of *L. helle* were sampled at 30 localities scattered over seven mountain groups in its Western European distribution area (Pyrenees, Massif Central, Jura, Madeleine mountains, Vosges, Ardennes-Eifel and the Westerwald) (Fig. 1, Table 1). Sampling was carried out from the beginning of May to the end of July in 2005–2007. The individuals were netted in the field and one leg per individual was removed and stored in 100% ethanol until analysis. To avoid recaptures of individuals, all sampled butterflies were marked on the wing with a permanent pen before they were released.

We selected five polymorphic microsatellites (LheF12, LheB06, LheE12, Lhe03, and Lhe14), which were developed for this species (Habel et al. 2008). The forward primer of each pair was 5' end-labelled with the fluorescent phosphoramidites FAM (LheE12 and LheB06), HEX (Lhe03) and TET (Lhe14 and LheF12). Details of the analytical procedures are described in Habel et al. (2008) and Finger et al. (2009).



**Fig. 1** Geographic locations of the 30 *Lycaena helle* sample sites in the Western European study area. Numbers correspond to localities given in Table 1. Dark grey areas mark elevations over 500 m

Potential distortion of the results through stutter bands, large allele drop-out or null alleles (cf. Kimberly and Selkoe 2006; Selkoe and Toonen 2006) was tested using the programme Micro-Checker (Van Oosterhout et al. 2004). Allelic richness was calculated using FSTAT (Goudet 1995). Hierarchical genetic variance analyses (AMOVAs), observed and expected heterozygosities and tests on Hardy Weinberg equilibrium (HWE) and linkage disequilibrium were calculated with Arlequin 3.1 (Excoffier et al. 2005). AMOVAs were carried out using the microsatellite-specific analogous *R*-statistics based on allele lengths and the stepwise mutation model. This statistical approach makes use of the fact that alleles similar in length are more likely to share a common ancestor (i.e. have a higher probability of being identical by descent). The analyses comprised three hierarchical levels: within populations, among populations within regions, and among regions. A population phenogram

**Table 1** Sampling localities and number of sampled *Lycaena helle*

Area	Locality	Abbrev.	Altitude	Area (km <sup>2</sup> )	Individuals
Pyrenees (PY)	F-Porté-Puymorens	P1	1,038	13,497 (12,809)	18
MassifCentral (M)	F-Complains	M1	1,261	17,172	20
	F-Picheland	M2	1,186	(4,097)	20
	F-Col Chaumoune	M3	1,156		20
	F- Mareuge	M4	1,389		20
	F-La Godivelle	M5	1,240		15
Jura (J)	F-Bellelay	J1	1,130	13,575	9
	CH-Tramelan	J2	1,086	(8,476)	24
	F-Le BémontI	J3	959		20
	F-Le BémontII	J4	1,021		9
	F-Le Mémont	J5	484		20
	F-Le Bélbrieu	J6	902		20
	F-La-R.-Drugeon	J7	809		20
	F-La Vacronnaz	J8	642		20
	F-Bois d' Amont	J9	1,070		25
	F-Tourbière	J10	886		10
Madeleine Mts. (MM)	F-La Verrerie	MM	642	(73515)	7
Vosges (V)	F-Gérardmer	V1	784	11,464	8
	F-Les Hauts Viaux	V2	1,077	(3,691)	25
	F-Xonrupt	V3	959		10
	F-Retournemer	V4	1,019		20
Ardennes (A) and Eifel (E)	B-Witry	A1	410	19,127	26
	B-Pisserotte	A2	493	(8,429)	24
	D-Klengelbachtal	A3	452		21
	D-Rohrvonn	E1	533		30
	D-Kolvenderbach	E2	485		21
	D-Frauenkron I	E3	568		23
	D-Mooshaus	E4	578		19
Westerwald (WW)	D-Lippe	W1	594	18,674	21
	D-Hof	W2	215	(11,432)	15

Abbreviations: *B* Belgium, *D* Germany, *F* France. Numbers of sites are as in Fig. 1. Size of area showing potential suitable habitats obtained from CEM (see text for further details); first value greater than a Maxent value of 0.50, second value greater than a Maxent value of 0.75 in paranthesis

using the neighbour joining algorithm (Saitou and Nei 1987) based on pairwise Cavalli-Sforza and Edwards (1967) distances was constructed with TREEMAKER. Node support was assessed by means of 1,000 bootstrap replicates. Furthermore, STRUCTURE (Pritchard et al. 2000) was used to cluster populations into groups to test the eight geographically isolated areas. Burn-in and simulation lengths were 100,000 and 500,000, respectively.

Population differentiation was tested using a *G* test based on allele frequencies, using Genepop (Raymond and Rousset 1995, Rousset 2008). Isolation by distance was tested by means of a Spearman test performed on half-matrices of pairwise

geographic distances (logarithmic scale) and the ratio  $F_{ST}/(1-F_{ST})$ , using  $F_{ST}$  between pairs of populations (Rousset 1997). These computations were performed with Genepop007 (Rousset 2008), on both the total data set and on a partial data set with only the three loci, which did not show heterozygote deficiency.

Alleles which exclusively occurred in one mountain group were identified. These alleles were neither present in any other population of our study area nor in populations of the complete European distribution area analysed (Habel et al. unpublished). Thus, the detected ‘private’ alleles are endemic for one obtained genetic group (and mountain region) and not for a single population sensu Slatkin (1985).

### 2.3 *Climate Envelope Model*

As shown in Waltari et al. (2007), Climate Envelope Models (CEM) provide a useful complement to genetic studies to predict ecological patterns of potential habitat distribution. In this study, 458 presence localities over the distributional range of *Lycaena helle* provided the basis for CEM computation. We used the CEM approach in order to assess the number and spatial distribution of recent and future climatically suitable habitats (cf. Hijmans and Graham 2006).

In order to assess potential suitable habitats, we used bioclimatic variables obtained from the WorldClim database (Hijmans et al. 2005; <http://www.worldclim.org/bioclim.htm>). Six variables were chosen: ‘annual mean temperature’, ‘maximum temperature of the warmest month’, ‘minimum temperature of the coldest month’, ‘annual precipitation’, ‘precipitation of the wettest month’ and ‘precipitation of the driest month’. These variables describe the climatic tolerance with respect to mean, minimum and maximum values of temperature and precipitation. They are likely to determine the distribution of our target species. We decided to extend the set of variables to six rather than applying three as used by Huntley et al. (2007) to model bird distribution, since these six variables reflect the ecological requirements of *Lycaena helle* more closely. This was shown by Bachelard and Descimon (1999) and our own data.

In order to assess the future potential distribution of *Lycaena helle*, we used a future anthropogenic climate change scenario developed by the National Center for Atmospheric Research (NCAR) Community Climate Model Version 3 (CCM3) as described by Govindasamy et al. (2003) and Duffy et al. (2003). This climate change scenario assumes a doubling of the CO<sub>2</sub> concentration by the year 2100 (Govindasamy et al. 2003; Duffy et al. 2003). All climatic data were down-scaled to a 2.5° spatial resolution, corresponding to a spatial resolution of about 3 × 3 km at 50°N.

To compute the CEM, we used Maxent 3.2.1 (Phillips et al. 2006, <http://www.cs.princeton.edu/~schapire/maxent/>), a machine-learning algorithm, which was shown to reveal better results than most other algorithms (e.g. Elith et al. 2006).

The default settings were used for the convergence threshold ( $10^{-5}$ ) with a maximum number of iterations (10,000). A logistic output format was used, whereby Maxent values range from 0 (not suitable) to 1 (very suitable) in a linear relationship. The software automatically selects suitable regularisation values to reduce overfitting (Pearson et al. 2006). For model testing, 75% of the occurrence points were used for model computation, and 25% were used as test points. The Area Under the Curve (AUC) was used to evaluate the model, based on the Receiver Operation Characteristics (ROC) curve as suggested by Phillips et al. (2006).

### 3 Results

#### 3.1 Genetic Analyses

The five microsatellite loci yielded between 16 and 54 alleles (mean:  $32.2 \pm 8.8$  sd), with a total number of 152 alleles over all loci and populations. No linkage disequilibrium was observed for any pair of loci after Bonferroni correction. Therefore, further analyses were performed on multi-locus data from all five microsatellites. After Bonferroni correction, significant deviations from HWE due to a heterozygote deficit were detected for the loci LheB06 and Lhe14. Using Micro-Checker (Van Oosterhout et al. 2004), we detected a strong signal for null-alleles for these two loci, but there was no indication of errors due to stutter bands or large allele drop-out for any locus. Thus, the HWE deviations at the loci LheB06 and Lhe14 could (at least partly) be due to the presence of null alleles. Values for allelic richness, observed and expected heterozygosity are given in Table 2.

The overall molecular variance among populations was 0.264 ( $p < 0.0001$ ) and 0.204 were found within populations ( $R_{IS}$ ). All populations were subdivided into geographic groups following the orographic structure of the study area (for classification see Table 1). The genetic differentiation among the seven entities was strong ( $R_{CT}$ : 0.203,  $F_{CT}$ : 0.146,  $p < 0.0001$  in both cases) all data are given in Table 3. A neighbour joining phenogram based on genetic distances (Cavalli-Sforza and Edwards 1967) revealed separate clusters corresponding with the respective mountain areas (Massif Central, Jura South, Jura North, Vosges, Ardennes-Eifel and Westerwald); the single populations from the Pyrenees and the Madeleine mountains are as strongly distinguished from all other samples as these clusters. Bayesian structure analysis of the data obtained for *Lycaena helle* individuals clearly discriminate the individuals into the same disjunct genetic groups mirroring the mountain areas. Within these groups, the degree of differentiation varies considerably. The Jura splits into two genetic groups – Southern and Northern – both of which were supported by Bayesian structure analysis with only few outliers. These two groups are also clearly discriminated by the neighbour joining phenogram (Fig. 2).

**Table 2** Parameters of genetic diversity for all analysed populations of *Lycæna helle* in the West European study area: mean number of alleles (A), allelic richness (AR), percentage of expected heterozygosity ( $H_e$ ), observed heterozygosity ( $H_o$ ) and private alleles (a: frequency, b: numbers)

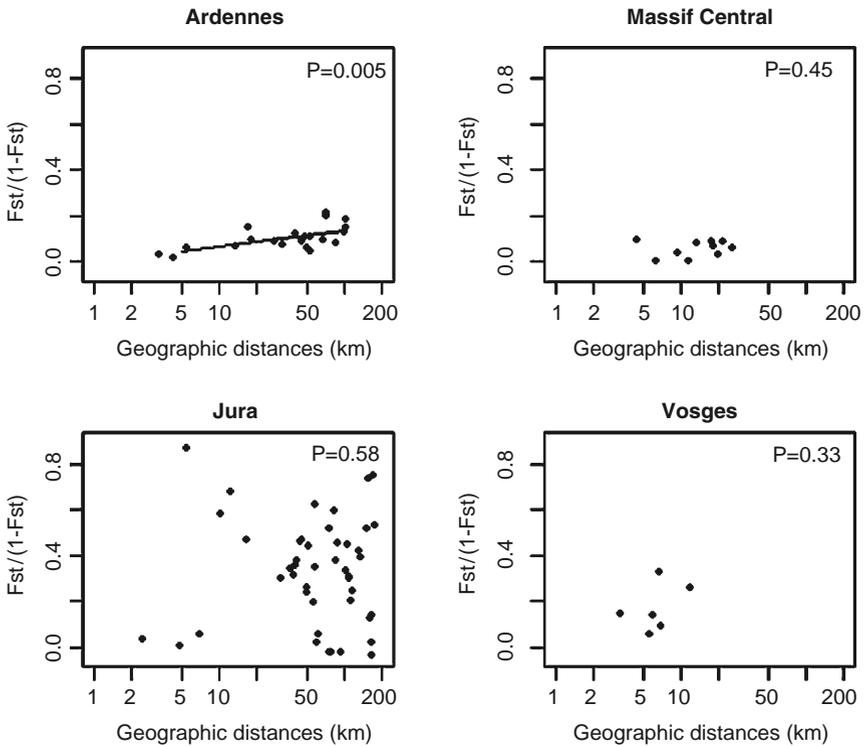
Area	Abbrev.	A	AR	$H_o$ [%]	$H_e$ [%]	Private alleles <sup>a</sup> [%]	Private alleles <sup>b</sup>
Pyrenees (PY)	P1	6.2	4.28	0.76	0.56	0.52	3.28
Massif Central (M)	M1	7.4	4.28	0.71	0.52	0.40	4.60
	M2	6.8	4.04	0.68	0.53	0.21	3.28
	M3	7.0	4.17	0.70	0.48	0.24	2.63
	M4	6.2	4.32	0.74	0.50	0.36	1.31
	M5	7.2	4.86	0.79	0.57	0.20	3.95
Mean (± s.d.)		6.92 (±0.46)	4.33 (±0.31)	0.73 (±0.04)	0.53 (±0.03)	0.38 (±0.09)	3.2 (±1.26)
	Northern Jura (J)	4.8	3.93	0.69	0.53	0	0
	J2	7.6	4.39	0.71	0.54	0.03	1.32
	J3	7.2	4.56	0.75	0.51	0.02	0.65
	J4	5.8	4.56	0.71	0.61	0	0
	J5	5.0	3.49	0.62	0.39	0	0
Mean (± s.d.)		6.8 (±1.17)	4.40 (±0.43)	0.72 (±0.04)	0.42 (±0.08)	0.03 (±0.01)	0 (±0.47)
	Southern Jura (J)	6.8	4.40	0.72	0.42	0	0
	J7	10	5.67	0.83	0.56	0.13	3.28
	J8	7.0	4.51	0.82	0.62	0.29	1.97
	J9	6.0	3.87	0.68	0.52	0.59	2.63
	J10	5.2	3.88	0.75	0.60	0.23	3.95
Mean (± s.d.)		7.00 (±1.82)	4.46 (±0.73)	0.76 (±0.06)	0.54 (±0.08)	0.32 (±0.19)	2.97 (±0.84)
	Madeleine Mts. (MM)	5.0	3.66	0.68	0.49	0.09	1.31

Vosges (V)	V1	3.2	2.78	0.49	0.36	0	0
	V2	3.6	2.80	0.51	0.39	0	0
	V3	3.4	2.87	0.52	0.42	0	0
	V4	3.6	2.91	0.58	0.43	0	0
Mean		3.45	2.84	0.53	0.40	0	0
(± s.d.)		(±0.19)	(±0.06)	(±0.04)	(±0.03)	(±0.0)	(±0.0)
Ardennes (A) and	A1	10.8	5.40	0.79	0.71	0.16	3.28
Eifel (E)	A2	7.0	4.35	0.74	0.60	0.25	1.97
	A3	7.0	4.18	0.73	0.67	0.22	1.97
	E1	8.0	4.83	0.77	0.75	0.12	1.32
	E2	8.2	4.82	0.77	0.58	0.33	2.63
	E3	8.0	4.66	0.74	0.57	0.20	2.63
	E4	6.0	4.16	0.72	0.59	0.07	1.31
Mean		7.86	4.63	0.76	0.65	0.19	2.16
(± s.d.)		(±1.51)	(±0.44)	(±0.03)	(±0.07)	(±0.08)	(±0.73)
Westerwald	W1	6.4	4.11	0.70372	0.55	0.09	1.32
(WW)	W2	5.2	3.79	0.675212	0.64	0.09	1.32
Mean		5.80	3.95	0.69	0.59	0.09	1.32
(± s.d.)		(±0.85)	(±0.23)	(±0.02)	(±0.06)		



**Table 3** Non-hierarchical variance analysis of *Lycaena helle* in five regions. All  $p < 0.001$

Region	$F_{ST}$	$R_{ST}$	$F_{IS}$	$R_{IS}$
Ardennes-Eifel	0.0854	0.0684	0.1342	0.2424
Massif Central	0.0467	0.1009	0.2657	0.3092
Northern Jura	0.0553	0.1065	0.2794	0.2975
Southern Jura	0.1078	0.2858	0.2700	0.2417
Vosges	0.0276	n.s.	0.1951	0.1195
Westerwald	0.0538	0.0761	0.1396	n.s.
All populations	0.1872	0.2643	0.1701	0.2040



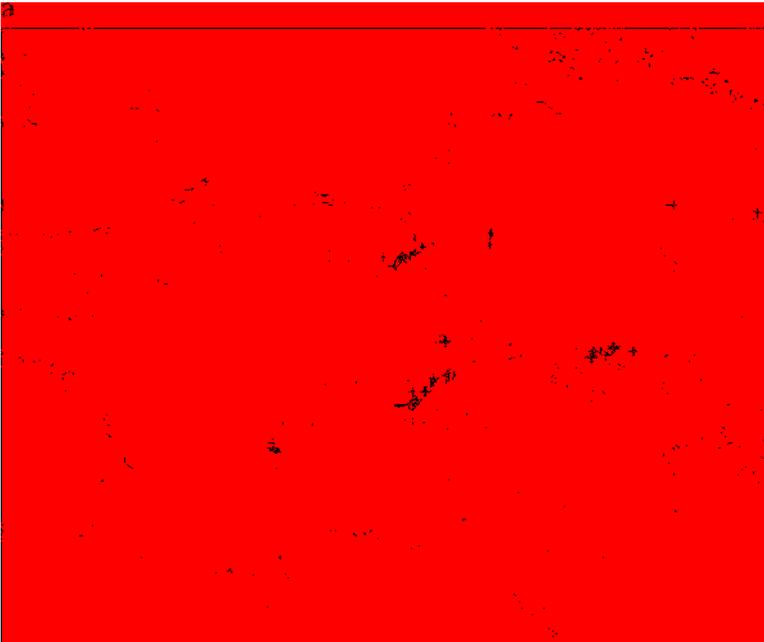
**Fig. 3** Isolation by distance in four regions studied. The ratio  $F_{ST}/(1 - F_{ST})$  as a function of the distances between populations (logarithmic scale)

Within each regional population, all subpopulations were significantly differentiated from each other ( $p < 0.001$ ). Isolation by distance was found to be significant only in the Ardennes–Eifel region (Fig. 3). No significant relationship between the number of private alleles or mean frequencies and area of potential suitable habitats (areas with Maxent values greater than 0.50 and 0.75) was detected.

### 3.2 *Climate Envelope Model*

The CEM suggests isolated potential distribution areas over the Western range of *Lycaena helle* under current climatic conditions. The areas with a predicted suitability of 0.75 or more (optimal regions) are mostly restricted to higher elevations (Fig. 4b). The areas of predicted suitability of at least 50% surround these mountainous areas. The areas of a predicted suitability higher than 75% are actually separated from each other by pessimal areas. The habitats which are potentially suitable from a climatic point of view also extend across major parts of the Northern and Southern Alps and most of the Balkan region. Distinct areas in Italy were also identified as potentially suitable. However, many of these areas (Italy, Balkans and the Southern and Western Alps) have not recently been colonised by *Lycaena helle*.

Applying the CCM3 scenario, the CEM suggests a strong decline of potentially suitable habitats, especially at the Western edge of the species' distribution area. Most of the recent areas of predicted suitability may disappear (Fig. 4c). Areas with a predicted suitability of 0.75 exclusively remain over parts of the Jura and the Alps. Areas with a predicted suitability of at least 0.50 remained in parts of the



**Fig. 4** Recent *L. helle* populations included in the models (a) modeled recent (b) and future distribution (c) of potential suitable habitats of *L. helle* in the Western European study area using Maxent Vers. 3.2.1. Presence of *L. helle* is shown by black crosses (Fig. 4a). Areas with a Maxent value of at least 0.50 are displayed in grey, areas with a predicted suitability of 75% or more are displayed in black

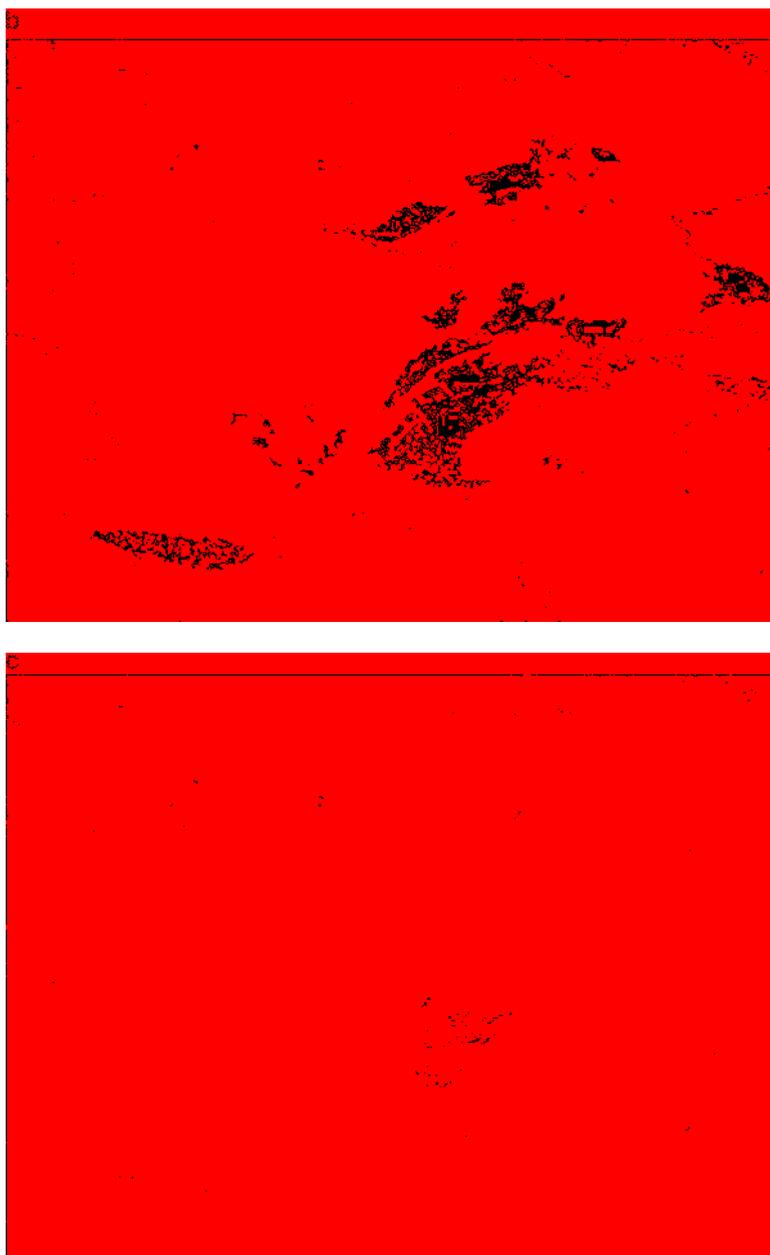


Fig. 4 (continued)

Massif Central, the Pyrenees and Vosges. Even over major parts of the Alps, potentially suitable habitats may largely disappear.

## 4 Discussion

The genetic data show: (1) eight genetic groups mostly corresponding with the orographic structures of higher elevations over our study area, (2) a high genetic differentiation among populations within these groups, (3) different levels of genetic diversities among the mountain regions and (4) private alleles, endemic for the mountain areas. The fact that the isolation by distance effect is significant only in the Ardennes region suggests that the other areas were subject to more complex colonisation and movement patterns than may be explained by distance *per se*; this pattern is opposite to the one shown for *Procllossiana eunomia* (also, a species feeding exclusively on *Polygonum bistorta*). In this species, the areas of high elevation (Pyrenees and Cantabrian mountains) show a high isolation by distance effect compared with areas of lower elevation as the Ardennes (Nève et al. 2008).

### 4.1 Historical Range Modifications

The present distribution pattern of *Lycaena helle* reflects demands for cool climatic conditions. From this we can assume a historically wide distribution over the major lowlands of Central Europe. We hypothesise two possible scenarios for the species' historical range modifications: (1) a colonisation of Central Europe from extra-Mediterranean and Eastern European refugia or (2) a wide distribution even during the glacial period. In both scenarios, a restriction of a former distribution range takes place. The first scenario would be in accordance with the butterfly *Procllossiana eunomia*, which represents similar ecological requirements (Nève 1996). However, the second scenario cannot be ruled out as fossil records (e.g. from pollen, charcoal fragments and the elytra of beetles, Huntley and Birks 1983; Coope 1994; Willis and van Andel 2004) support the idea that climatic conditions and basic habitat requirements for *Lycaena helle* (such as the larval food plant) were present during this period, and a CEM suggests a larger area of potential suitable habitats for *Lycaena helle* during the glacial period (Habel et al. unpublished data).

### 4.2 Patchily Distributed and Genetically Differentiated

In most of the populations analysed we detect a high genetic variability, which might reflect a former wide distribution for this species – or at least interconnected populations within each mountain area. In general, isolated population remnants show

reduced genetic diversity as a result of genetic erosion (Lesica and Allendorf 1995), as shown for other butterflies like *Parnassius apollo* (Habel et al. 2009), *Parnassius smintheus* (Keyghobadi et al. 2005), *Speyeria idalia* (Keyghobadi et al. 2006), *Proclossiana eunomia* (Nève et al. in press), and the ground beetle *Carabus auronitens* (Drees et al. 2008). Obviously, the present mountainous retreats of Central Europe left sufficient space for intact metapopulations of *Lycaena helle* to maintain this high genetic diversity to the present day. The phenomenon of high genetic diversity despite long-term isolation was also shown for other relict organisms, which maintained their genetic variability through intact population networks within isolated areas (cf. Baali-Cherif and Besnard 2005). As the detected private alleles do not appear in further populations over the entire European distribution area (Habel et al. unpublished data), their evolution should have taken place in most cases after the species colonised these mountain areas during the post-glacial warming.

The strong genetic differentiation among mountain areas coincides with the orographic structure of the study species and with the optimal areas (potential suitable habitats surpassing a Maxent value of 0.75) of the CEM. Less suitable regions (potential suitable habitats with a Maxent value of at least 0.50) enclose these hotspots. Most of the classified mountain areas are not linked with neighbouring areas by habitats of potential suitability (not even areas with a Maxent value of at least 0.50). As many studies reveal the importance of habitat size to maintain genetic information, we analysed all genetic diversities in respect to area size of suitable habitats obtained from the Ecological Niche Model and could not find any correlations.

Even within each mountain area, we detected a strong genetic differentiation among neighbouring populations. Detected private alleles are often restricted to a single population of a mountain group, which underpins restricted or absent geneflow among populations of *Lycaena helle* within mountain massifs. Within the Ardennes-Eifel, we detected a significant correlation between the level of genetic differentiation among populations and the geographical distance between them, which suggests a restricted geneflow among populations between these two adjoining areas, clustering into two genetic groups with only a few outliers (Finger et al. 2009). For the Jura massif the statistic analysis revealed a significant split into a Southern and Northern group and is in accordance with two morphologically characterised subspecies (Meyer 1982). These two morphological and genetic character sets give results which allow us to conclude that the two groups of populations inhabiting the Jura massif represent two distinct Evolutionarily Significant Units (ESU) sensu Moritz (1994). However, these deep structures are probably not the result of recent habitat isolation but of historic species separation into distinct refugia, as described for the snail *Trochulus villosus* in the same area (Dépraz et al. 2008).

The CEM suggests strongly fragmented potential suitable areas for *Lycaena helle*, corresponding with the higher elevations of our study area. The obtained pattern coincides with the recent genetic and distribution situation of this species (Drees and Pretschner 2003). The CEM projection onto a future climate change scenario suggests a severe decline of most potential suitable habitats over Western

Central Europe, restrictions to higher elevations of the Jura and Alps and an increase in the habitat fragmentation of the remaining areas. Similar changes in distribution patterns have been suggested for birds occurring in the same climatic zone as *Lycaena helle*, such as Hazel Grouse (*Bonasa bonasia*) or the Pygmy owl (*Glaucidium passerinum*) (Huntley et al. 2007).

### 4.3 Future Habitat Decline and Loss of Genetic Uniqueness

Projecting the CEM onto the CCM3 climate change scenario, our model suggests a complete loss of climatically suitable habitats for *Lycaena helle* on its Western distribution border, except for a few exclaves of potentially suitable areas over the Pyrenees, the Massif Central, the Jura and parts of the Alps. This implies two effects: (1) an immediate range loss involving both population numbers and their genetic diversity and (2) more frequent local extinctions of populations due to stochastic processes by increasing habitat fragmentation (cf. Hanski 2005). The direct loss of distinct population groups would imply the loss of a large part of the genetic diversity, including numerous private alleles, which are exclusively present in these populations and will not be preserved in any other populations over the entire European range (Habel et al. unpublished data).

The interpretation of the CEM results merits some theoretical considerations. Although it is widely accepted that species' climatic envelopes behave conservatively especially over relatively short time periods (e.g. Peterson et al. 1999; Wiens and Graham 2005), species theoretically have the *ability* to adapt to climate change and thus compensate range loss in terms of their potential distribution. This depends on the succession of generations driven by natural selection, ecological potency and change rates of the environment (e.g. Holt and Gomulkiewicz 2004). Furthermore, species inhabiting large areas are *less* vulnerable than species with smaller ranges because of a greater genetic diversity and hence a greater probability of successful adaptations can be expected (e.g. Holt and Gomulkiewicz 2004, Parmesan 2006). In our study, the remaining habitats will become even more isolated from each other than they are today. This situation *may* enforce negative effects, such as genetic drift and the loss of allelic richness and uniqueness in the near future.

## 5 Conclusions

Combining the advantages of both methods – molecular analysis and Climate Envelope Modelling – creates a complete picture of the status of a species. While genetic data give direct evidence of a species' history and its recent situation over different spatial scales, the data of a CEM show the present distribution and its future tendencies. Thus, both methods give information on different spatial and

temporal scales and form a complete picture of a species, and in our case of a species which is representative of a large number of cold-adapted species.

**Acknowledgement** We acknowledge a grant from the Ministry of Culture, Education and Sciences Luxemburg (grant number BFR-05/118), the Natural History Museum Luxembourg. Thanks go to all lepidopterologists who helped us to collect the species and/or showed us suitable sites. We also thank Aline Finger (Zürich, Switzerland) for the laboratory work and Thomas Schmitt (Trier, Germany) for critical comments on a draft version of this manuscript.







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# Relict Species Research: Some Concluding Remarks

Jan C. Habel, Thomas Schmitt, and Thorsten Assmann

Relict species or populations mostly occur in small and isolated habitats and are remnants of wider distributions in the past. The recent situation of such populations, which have often been scattered along the periphery of the distribution range over long time periods, has led to independent evolutionary processes. These are reflected today in morphologically and genetically unique characteristics that occur exclusively in these populations. Such evolutionary lineages caused by long-term isolation are often described as “evolutionary significant units”; the theory emphasises the distinctiveness of locally restricted phenological, morphological, and/or genetic units. Furthermore, these differentiation processes represent an important evolutionary potential, which distinguishes relict populations from the populations of the main distribution. This evolution in relict populations may in some cases be of high value for the future adaptiveness of the species concerned. On the other hand, very isolated relict populations often also suffer from repeated bottlenecks and the negative effects of genetic drift which enforce the evolution of local uniqueness, but cause losses of genetic diversity on the taxon and intraspecific level. Thus, ecological selection acts much more strongly and therefore more quickly in small and isolated remnant populations than in core populations. Furthermore, populations at the edge of the species’ distribution are often exposed to ecological conditions that are different from those in the core area. This selection under different habitat conditions can lead to new allele combinations essential to survival under conditions which differ from those of the core area; they may therefore be relevant for responses to the challenge of future climate changes. Following the contribu-

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tions presented in this book, we conclude that peripheric populations are of particular importance for conserving the complete evolutionary and adaptive potential of species and their intraspecific variability in terms of the Convention on Biological Diversity.

We hope that this compilation of contributions on relict species and populations is of interest for conservationists and biologists, as many aspects of the fields conservation biology, evolutionary biology, ecology, paleontology, modelling, and conservation politics are highlighted using a variety of methods, including molecular genetics, morphometry, and habitat, as well as climate envelope modelling, to explain the complexity of relict species against the background of climate modifications in the past, present, and future.