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

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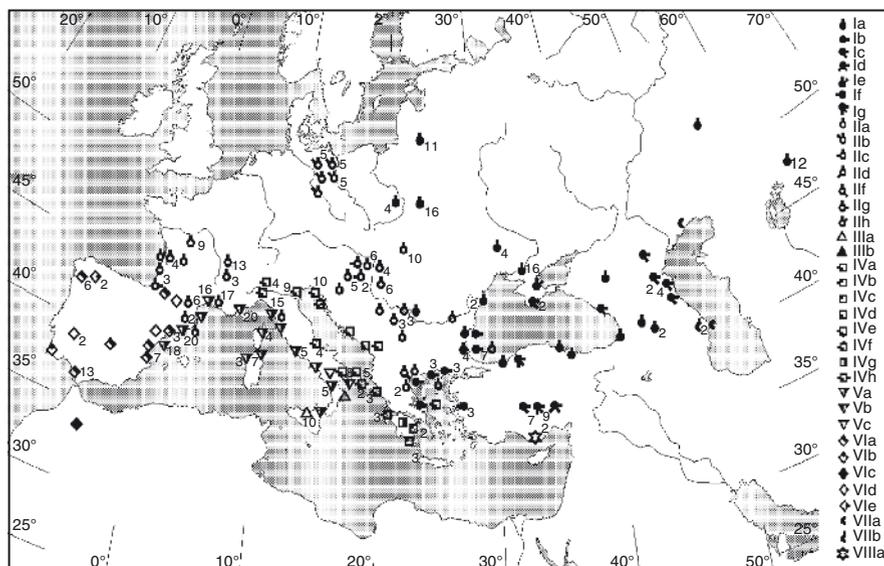
Fig. 1 Geographical distribution of *Emys haplotypes* (only natural populations). Some rare haplotypes are not shown. Note that only haplotypes of groups I and II occur naturally North of the Alps

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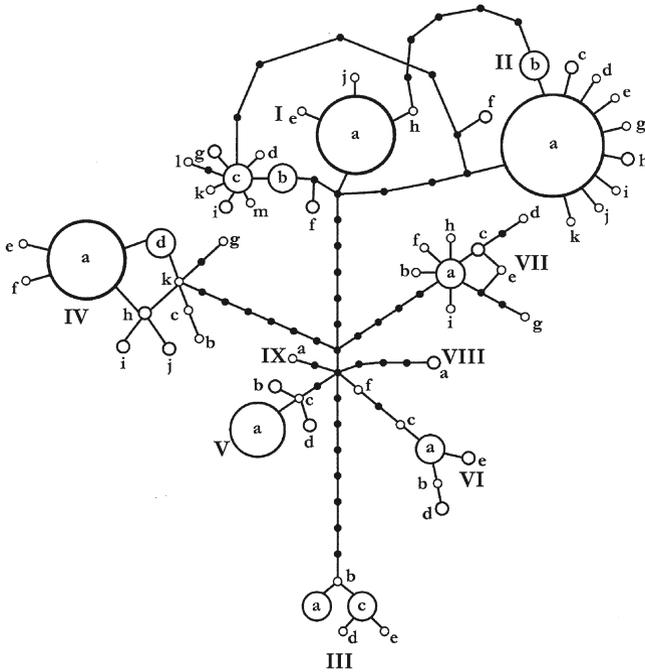
Fig. 2 TCS parsimony network of the *Emys* haplotypes. Haplotype group III = *Emys trinacris*. *Open circles* symbolize known haplotypes, *circle* size corresponds to the frequency of haplotypes as detected; *dots* on lines show single substitution steps between haplotypes (most parsimonious connection)

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 VId, 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 (Nettmann 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 aglis 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
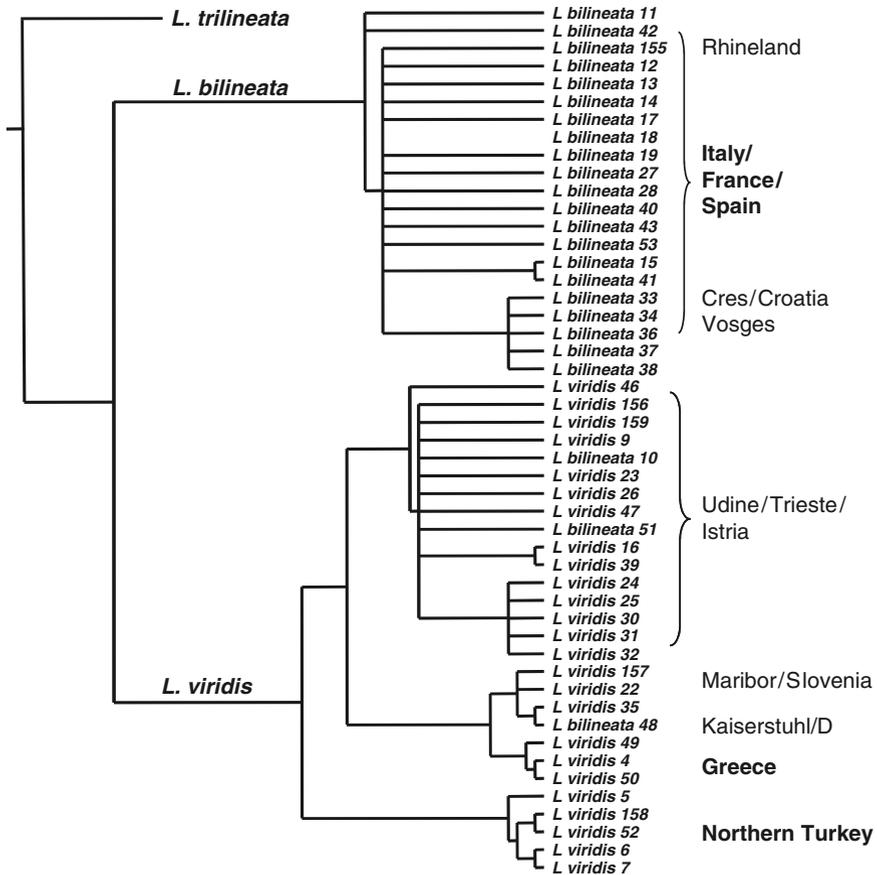
Fig. 3 Maximum parsimony tree for Green lizards, derived from mitochondrial cytochrome b sequences. Note that several individuals were initially determined as *L. bilineata* (from Trieste, Italy, and Kaiserstuhl, Germany), but have haplotypes of *L. viridis*, whereas lizards from the Croatian island 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
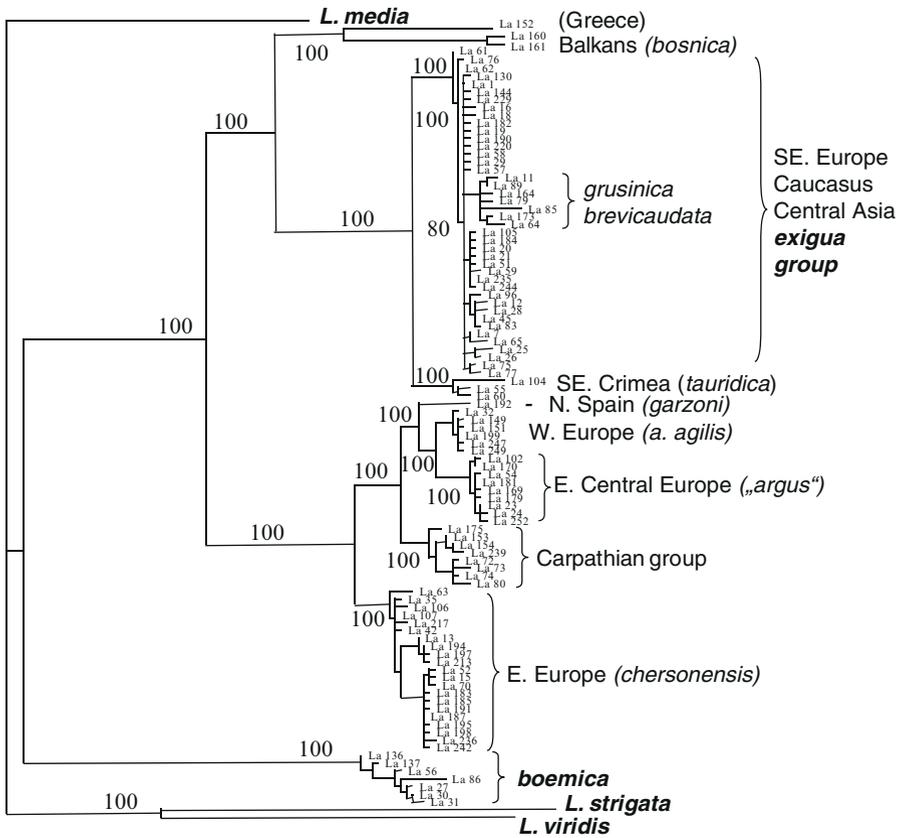
Fig. 4 Maximum Parsimony phylogram (consensus of 300 equally parsimonious trees) of the *Lacerta agilis* complex, derived from mitochondrial cytochrome b sequences. *L. media*, *L. strigata,* and *L. viridis* are used as outgroups. Bootstrap values indicated. The *L. exigua* group unites subspecies from 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 finger-prints, 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
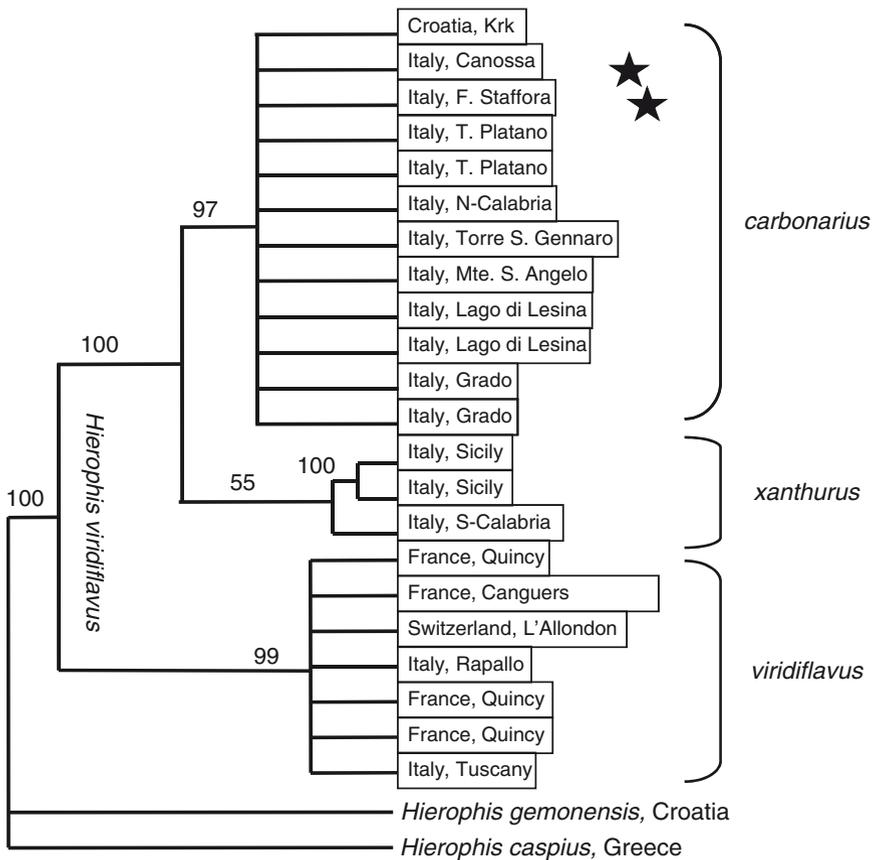

Fig. 5 Phylogeography of *Hierophis viridiflavus* derived from cytochrome b sequences. Maximum Parsimony tree with bootstraps values for the main haplotype groups. *=Populations East of the Apennines, in which alleles of the Western group were recorded by ISSR-PCR (putative hybrids)

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 PindosMountains 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
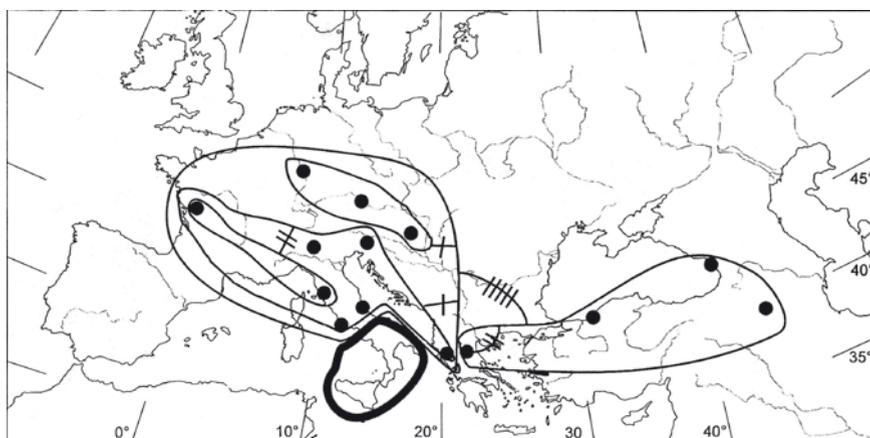

Fig. 6 Phylogeographic grouping of Aesculapian snakes (*Zamenis longissimus* and **Z. lineatus**) derived from mitochondrial cytochrome b sequences

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 Morrocco 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).
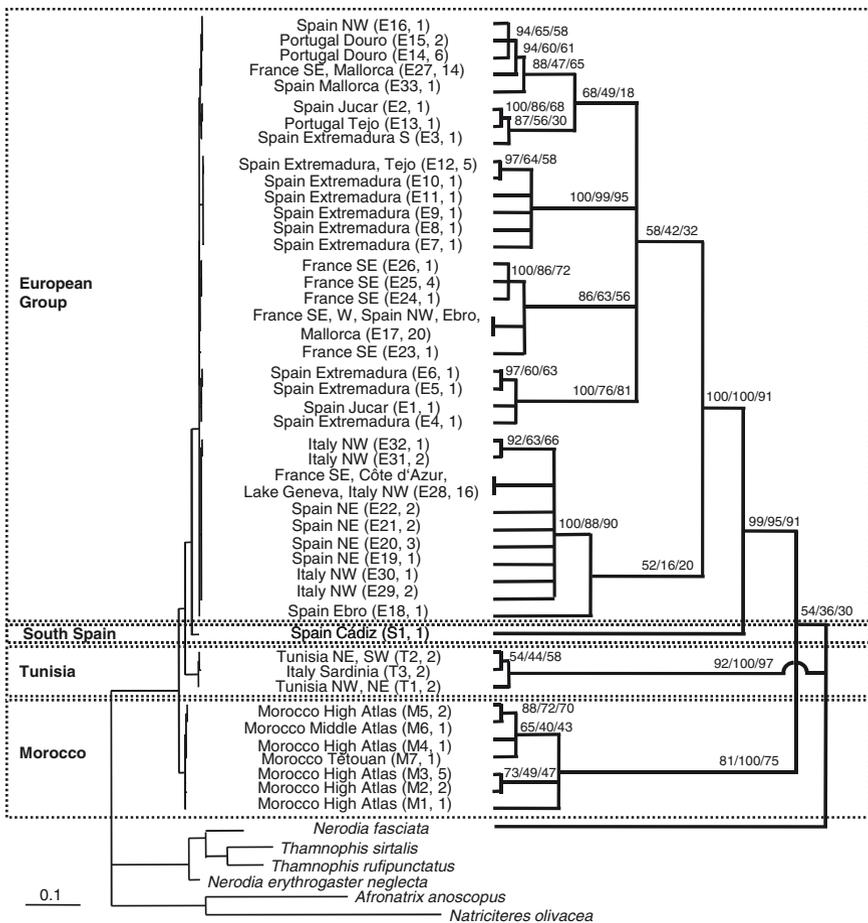

Fig. 7 Phylogenetic trees of *N. maura* reconstructed from cytochrome b haplotypes, *left*: maximum likelihood phylogram, *right*: Bayesian cladogram. Sequences from *N. natrix* and *N. tessellata* have been used for outgroup rooting. Support values are indicated in the order Bayesian/ML bootstrap values/MP 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 occuring 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.
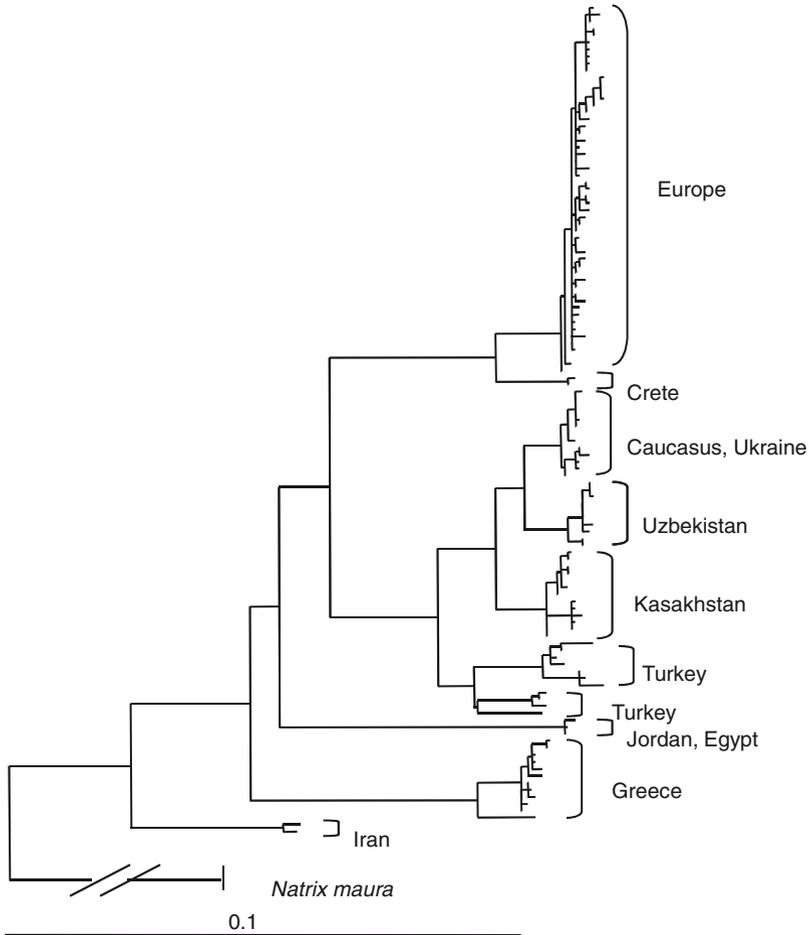
Fig. 8 Maximum Likelihood Phylogram for *Natrix tessellata,* based on complete cytochrome b sequences. 9–10 different haplotype groups can be distinguished. *Natrix maura* served as outgroup

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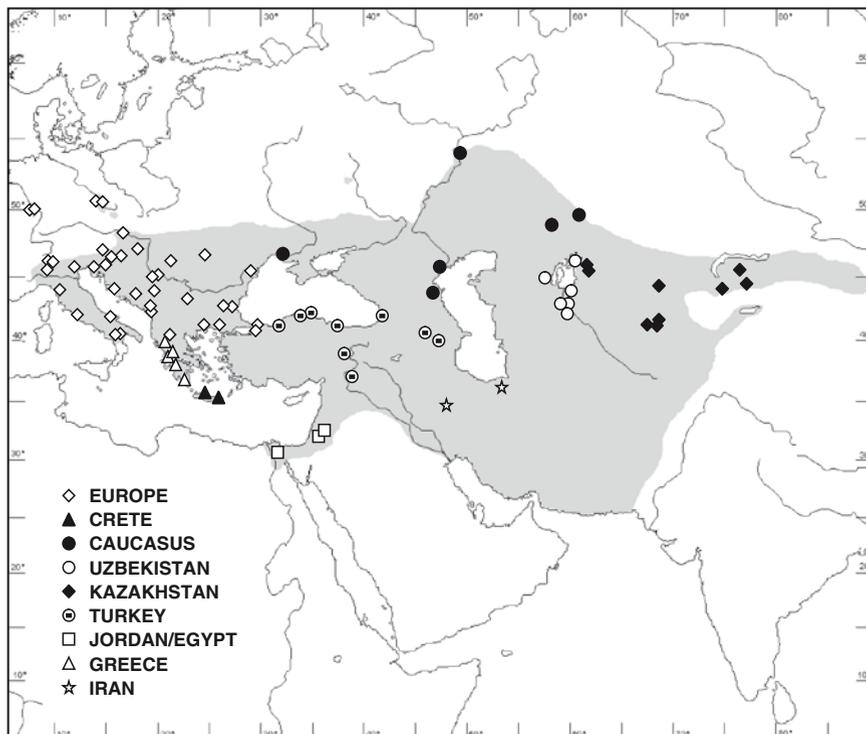
Fig. 9 Geographical distribution of haplotype groups in *Natrix tessellata,* modified from Guicking et al. (2009.)

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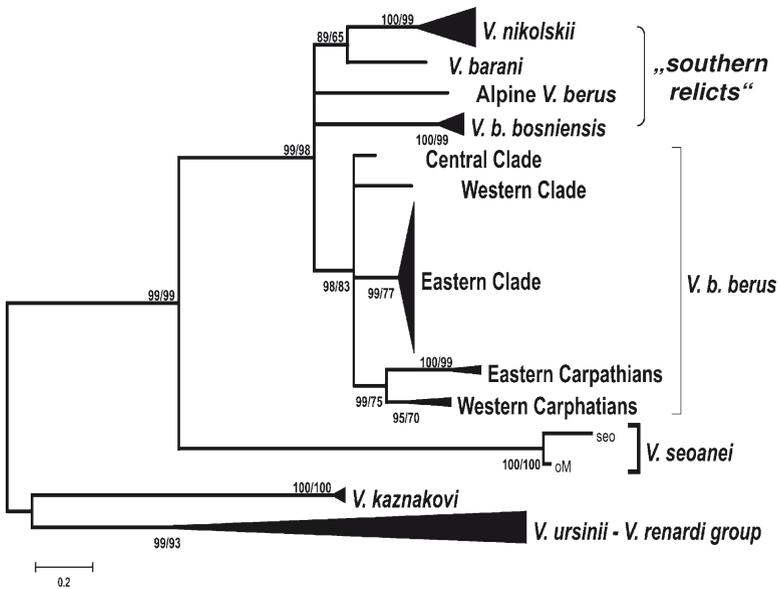
Fig. 10 Maximum Parsimony strict consensus tree of *Vipera berus* and relatives derived from cytochrome b haplotypes (1,003 bp). Bootstraps values indicated for Maximum Parsimony and Maximum Likelihood. *Vipera kaznakovi, V. renardi and V. ursinii* were used as outgroups

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 (Gliemeroth 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 cytochome 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
Emys	1.4–1.7[a]	3–4	1.4–3	Lenk et al. (1999)
Lacerta agilis	6.5–7.3[b]	2.6–2.9	0.7–2.4	Kalyabina et al. (2001)
L. viridis complex	6.6–8.4[c]	2.6–3.4	0.5–2.5	Böhme et al. (2006)
Hierophis	10.5[d]	8–9	0.9–2.7	Nagy et al. (2002, 2004)
Zamenis (*Elaphe*)	7[e]	5–7	0.5–1	Lenk et al. (2001)
Natrix	18[f]	13–22	2–8	Guicking et al. (2006a)
Vipera berus group	5[g]	4	1.1–1.6	Kalyabina-Hauf et al. (2004b)

[a]Distance between *E. orbicularis* und *E. trinacris*
[b]Distance between *L. agilis* sspp. and *L.* (*a.*) *boemica* (assuming species status)
[c]Distance between *L. viridis* and *L. bilineata*
[d]Distance between *H. viridiflavus* and *H. gemonensis*
[e]Distance between *Z. longissimus* and *Z. lineatus*
[f]Distance between *N. natrix* and *N. tessellata*
[g]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
Emys	several	several	several	several	1	North African
Lacerta agilis	1	–	2	1	several	Carpathian
L. viridis complex	–	2	several	1	–	
Hierophis viridiflavus	–	several	–	–	–	
Zamenis longissimus	–	2	2	?	?	
Natrix maura/ N. tessellata	several	?	2	several	several	North African West Asian
Vipera berus group	1(*V. Seoanei*)	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 opti-mum 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 conser-vation categories. Yet, if the available genetic data are considered, their extinc-tion 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, signifi-cantly 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 dis-tinct. Pleistocene relict populations have often been isolated by ecological change (sometimes with human interference, as the aquatic *N. tessellata* in the desertifi-cated 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* popula-tion 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
Emys orbicularis complex	*E. trinacris* Sicily	*E.o.persica* Caspian Area *E.o.*ssp. S. Anatolia	*E. orbicularis* North Africa *E.o.orbicularis* Turkey	*E.o.orbicularis* E. Germany, Lithuania, Kazakhstan
Lacerta viridis complex	*L. bilineata* ssp. S. Italy, Sicily *L. viridis* ssp.Balkans	*L. viridis* ssp.Turkey	*L. bilineata* Balkans, Cres Isl.	*L. bilineata* W. Germany *L. viridis* E. Germany
Lacerta agilis complex	*L.a.tauridica* Crimea	*L.(a.)boemica* Caucasus *L.a.bosnica* Balkans *L.a.garzonii* Pyrenees	*L.a.chersonensis* Carpathians	*L. agilis* Britain, Sweden
Hierophis viridiflavus	*H.v.xanthurus* S. Italy, Sicily			
Zamenis longissimus complex	*Z. lineatus* S. Italy, Sicily			*Z. longissimus* Germany, Czech Rep.
Natrix maura	undescribed North Africa	undescribed Andalucia	*Natrix maura* Southern Spain	
N. tessellata	undescribed Middle East	undescribed Greece, Middle East	*N. tessellata* Crete, Aral Sea region	*N. tessellata* W. Germany, Czech Rep.
Vipera berus complex	*V.b.bosniensis* Balkans	*V. barani* Turkey *V. nikolskii* Black Sea area *V. seoanei* Pyrenees	*V. berus* Alpine Region *V. berus* Greece *V.b.berus* 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 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 contributea 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.

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