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Phylogeography and Pleistocene refugia of the adder (*Vipera berus*) as inferred from mitochondrial DNA sequence data

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Abstract

In order to contribute to the debate about southern glacial refugia used by temperate species and more northern refugia used by boreal or cold-temperate species, we examined the phylogeography of a widespread snake species (*Vipera berus*) inhabiting Europe up to the Arctic Circle. The analysis of the mitochondrial DNA (mtDNA) sequence variation in 1043 bp of the cytochrome *b* gene and in 918 bp of the noncoding control region was performed with phylogenetic approaches. Our results suggest that both the duplicated control region and cytochrome *b* evolve at a similar rate in this species. Phylogenetic analysis showed that *V. berus* is divided into three major mitochondrial lineages, probably resulting from an Italian, a Balkan and a Northern (from France to Russia) refugial area in Eastern Europe, near the Carpathian Mountains. In addition, the Northern clade presents an important substructure, suggesting two sequential colonization events in Europe. First, the continent was colonized from the three main refugial areas mentioned above during the Lower-Mid Pleistocene. Second, recolonization of most of Europe most likely originated from several refugia located outside of the Mediterranean peninsulas (Carpathian region, east of the Carpathians, France and possibly Hungary) during the Mid-Late Pleistocene, while populations within the Italian and Balkan Peninsulas fluctuated only slightly in distribution range, with larger lowland populations during glacial times and with refugial mountain populations during interglacials, as in the present time. The phylogeographical structure revealed in our study suggests complex recolonization dynamics of the European continent by *V. berus*, characterized by latitudinal as well as altitudinal range shifts, driven by both climatic changes and competition with related species.

Keywords: colonization history, mitochondrial DNA, molecular phylogeography, snake, *Vipera berus*

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Introduction

The recolonization of Eurasian biota into landmasses that had been deserted during harsh glacial times has been a subject of intense study especially during the past 10 years, thanks to advances in molecular genetic techniques. The current distributions of temperate species are the results of multiple range shifts driven by past climatic changes. The temperate fauna and flora of Europe is generally considered to have survived glaciations in any of three peninsular refugia in the south of the continent, i.e. Iberia, Italy or Balkan, or further to the east, around the Caucasus Mountains. From these refugia, species expanded during more favourable climatic periods (interglacials and interstadials). This pattern has been corroborated by genetic data from several organisms studied to date (for reviews, see Hewitt 1996; Taberlet *et al*. 1998; Hewitt 1999, 2000, 2004). Challenging this model, recent investigations based on fossil and genetic data have suggested the presence of

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other, more northerly situated refugial areas located outside of southern European peninsulas, such as Hungary or near boundaries of permafrost (e.g. Bilton *et al*. 1998; Willis *et al*. 2000; Stewart & Lister 2001; Brunhoff *et al*. 2003; Haase *et al*. 2003; Palme *et al*. 2003).

The haplotypic and maternally inherited mitochondrial DNA (mtDNA) has become the molecular marker of choice for phylogenetic and phylogeographical studies in animals, and investigating mtDNA variation has provided valuable information for resolving intraspecific phylogeographies in several different species (e.g. Avise 2000). The noncoding control region (CR) has been the part of the mitochondrial genome most frequently targeted, as it generally provides sufficient variation for studies on an intraspecific level (Brown *et al*. 1979; Moritz *et al*. 1987). Commonly used mtDNA coding regions have been the cytochrome *b* (cyt *b*) gene and to a lesser extent the NADH dehydrogenase subunits (ND1 to ND6). In some cases, these genes have proved to be equally or more variable than the CR even though they are coding sequences and could be expected to be exposed to more intense selection pressure (Questiau *et al*. 1998; Crochet & Desmarais 2000; Randi *et al*. 2001). Therefore, several authors have recently questioned the applicability of the CR for intraspecific comparisons of diversity in some snake species, as the variation found was low (Ashton & de Queiroz 2001; Douglas *et al*. 2002).

The adder (*Vipera berus*) is the most widely distributed terrestrial snake species in the world, ranging throughout almost a third of the landmass of the Eurasian continent (Saint Girons 1980). It can be found from Scotland in the west to Pacific Russia in the Far East, and from Albania and Greece in the south to well north of the Arctic Circle (69°N) in Fennoscandia (see Fig. 1). Three subspecies are currently recognized of which *Vipera berus bosniensis* (Boettger 1880) occurs on the Balkan Peninsula and *Vipera b. sachalinensis* (Zarevsky 1917) is restricted to Sakhalin Island and the extreme east of Russia. The nominate form *Vipera b. berus* occurs throughout all other parts of the distribution range of the species. Being a cold-tolerant reptile, it is found in mountain areas at relatively high altitudes (1000–2600 m above sea level) in the southern extremes of its range (Gasc *et al*. 1997). As is suggested from the vast expanse of land populated by the nominate subspecies, this species exhibits a low level of geographically correlated morphological variation (Saint Girons 1978). To date, a number of studies have investigated the genetic variation of adders on local to regional scales (Stille *et al*. 1986; Carlsson & Tegelström 2002; Carlsson *et al*. 2004), but until now, no study has addressed the genetic variation of the adder across all of its geographical range.

The purpose of the present study is to characterize the genetic structure and colonization history of adders across the entire distribution range using mtDNA sequence data. The choice of a reptilian species adapted to northern environments could bring additional insights to the competing views of the current distribution ranges of wide spread species being populated purely from southern glacial refugia vs. predominantly from refugia in central Europe. Finally, the genetic data presented herein should partly remedy the lack of intraspecific phylogeographies of Palaearctic reptile species available to date.

Materials and methods

Sample collection

Samples from 80 specimens of *Vipera berus* distributed over 60 localities across the entire species distribution range (with a bias towards the presumed core areas of southern Europe; Fig. 1) and from one specimen of *Vipera seoanei* were obtained in the field or from existing collections (see Appendix). DNA was extracted either from tissue of frozen or preserved specimens, while sloughs were collected in the field. Blood was sampled from the caudal vein of living specimens (Bush & Smeller 1978) and stored in Queen's lysis buffer (Seutin *et al*. 1991) or ethanol. Total genomic DNA was extracted using either QIAamp DNA Mini Kit or DNeasy Tissue Kit (QIAGEN), or through a salt-extraction procedure (Paxton *et al*. 1996).

Genetic analysis

A 918-bp portion of the mtDNA CR was amplified and sequenced for all 80 sampled *V. berus* specimens. A selection of 50 of these individuals, representing most CR haplotypic diversity, was also sequenced for 1043 bp of the cyt *b* gene. After the alignment of three published snake sequences (Kumazawa *et al*. 1996, 1998) for purposes of primers design, a first fragment of the CR was amplified using the primers L16148VA (5′-TCTCTAGCCCCCAAAGCTAG-3′) and H16551VA (5′-AGCCAGAGGCCTTGGAAAGAG-3′). The numbers correspond to the position on the mtDNA molecule of *Dinodon semicarinatus* (Kumazawa *et al*. 1998). The second CR part, amplified using the primers L16571VA (5′-CTCTTTCCAAGGCCTCTGGCT-3′) and H690 (Kumazawa *et al*. 1996), was located on the duplicated CR present in advanced snakes (Kumazawa *et al*. 1996, 1998). These two copies of the CR were nearly identical in previously investigated advanced snakes (Kumazawa *et al*. 1996), and were identical in one adder examined (totalling 1101 bp for both CR copies; V. Helfer, unpublished). The cyt *b* gene was amplified with the primers L14724Vb (5′-GATCTGAAAAACCACCGTTG-3′), H15548Vb (5′-AAATAGAAAGTATCATTCTGGTTTAAT-3′), L15162Vb (5′-CTCCCATGAGGACAAATATC-3′) and H15914Vb (5′-CCAGCTTTGGTTTACAAGAAC-3′), designed from several published reptilian sequences

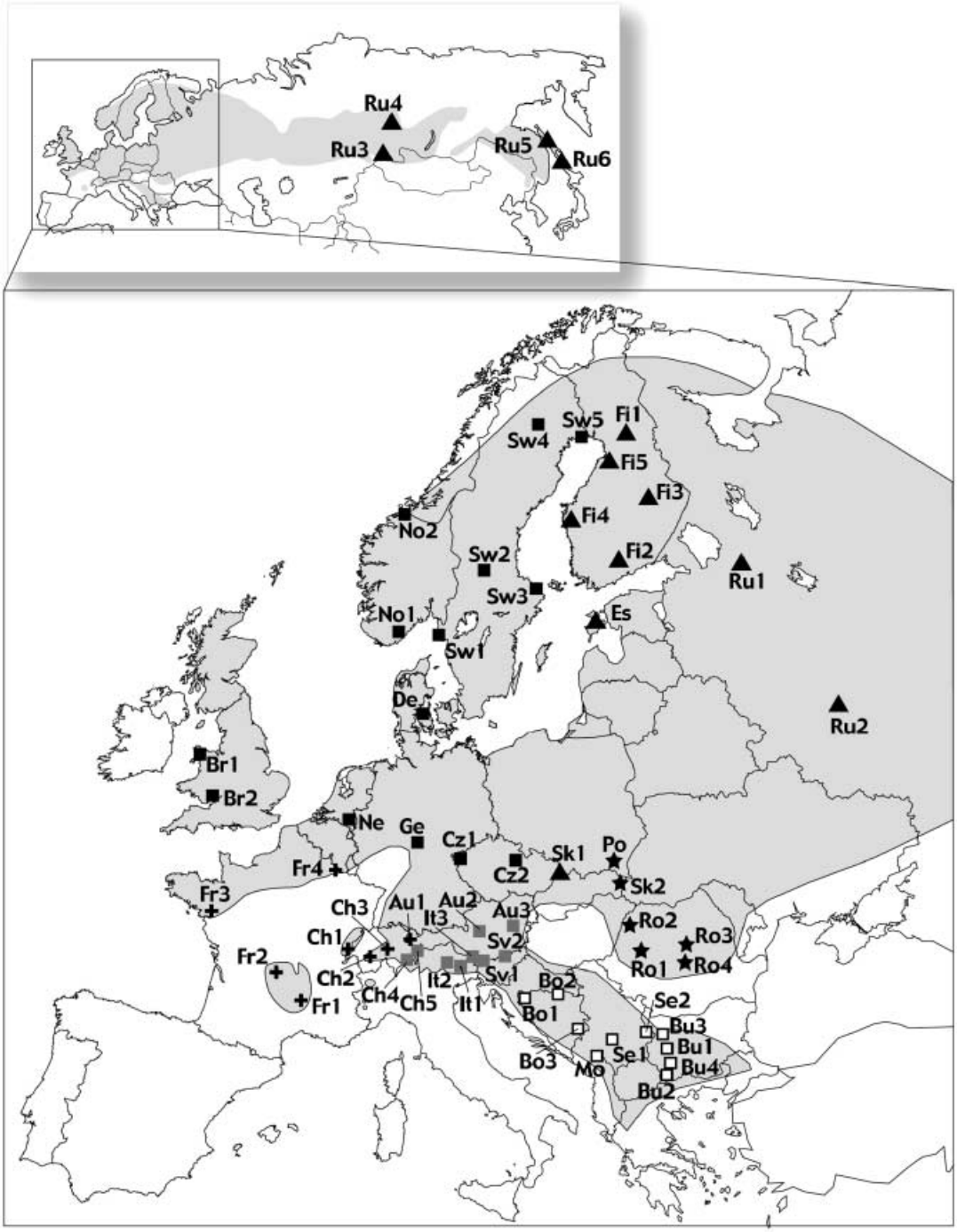

Fig. 1 Sampling localities for 80 adders from 60 localities analysed in the present report. Population code refers to the Appendix, and symbols correspond to phylogenetic affinities as shown in Fig. 2. Grey areas indicate present-day distribution of the species.

(Kumazawa & Nishida 1995; Kumazawa *et al*. 1996, 1998; Janke & Arnason 1997) and a partial cyt *b* sequence of *Vipera ursinii* deposited in GenBank (Accession no. AF182552). Polymerase chain reaction (PCR) was conducted in 25-μL volumes with 2 μL of DNA template, 1× PCR buffer (QIAGEN), 2 mg/mL of Q solution (QIAGEN), 2 mM of $MgCl_2$, 0.2 mM dNTPs, 0.5 μM of each primer and 0.5 U of *Taq* polymerase (QIAGEN). Amplification conditions consisted of 35–45 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 56 °C for the first portion of the CR and 59 °C for the second portion, and extension for 45 s at 72 °C. The PCR conditions for the cyt *b* were: 35–45 cycles

of 94 °C for 45 s, 50 °C for 60 s and 72 °C for 90 s. PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN). Cycle sequencing was performed with the primers H16551VA, L16571VA, H690, L14724Vb and H15914Vb in 7.5 μL containing 3–4 μL of amplified DNA, 0.1 μM of primer and 3 μL of ABI PRISM Dye Terminator cycle sequence Ready Reaction Kit (Perkin-Elmer). Water was added up to 7.5 μL. Reaction sequences were visualized on an ABI PRISM 377 automated DNA sequencer (Applied Biosystems). Sequences were deposited in GenBank (Accession nos: DQ185938–DQ186080).

Data analysis

Mitochondrial DNA sequences were aligned using the CLUSTAL method (Higgins *et al*. 1992). The possibility of saturation at first, second and third codon positions of the cyt *b*, as well as for transition and transversion substitutions at third codon position, was evaluated by plotting uncorrected pairwise sequence divergences against pairwise divergences corrected by the substitution model selected for the phylogenetic analyses (see below). Saturation in the combined CR and cyt *b* dataset was also tested by plotting the uncorrected distances against the corrected ones. To compare the relative rate of evolution between CR and cyt *b*, we plotted the uncorrected pairwise distances of CR vs. cyt *b* gene.

Phylogenetic analyses

The 50 samples of *V. berus* that had been sequenced both for the CR (918 bp) and the cyt *b* (1043 bp) were used for phylogenetic analyses. The sequences were analysed with PAUP* version 4.10b (Swofford 2002). We performed a partition homogeneity test (using 1000 replicates) in order to examine whether both analysed regions could be combined in a unique data matrix (Farris *et al*. 1995).

Maximum parsimony (MP) analyses were performed [heuristic searches with random stepwise addition for 1000 replicates and tree-bisection–reconnection (TBR) branch swapping options] with all indels considered as a new state (fifth base). Comparable results were obtained when indels were considered as missing data (data not shown). The combined data set was also analysed with maximum-likelihood (ML) and neighbour-joining (NJ) methods. Maximum-likelihood analyses were conducted with PHYML 2.4.4 (Guindon & Gascuel 2003) and NJ with PAUP*. For the ML and NJ analyses, a total of 56 substitution models were evaluated using MODELTEST version 3.6 (Posada & Crandall 1998) and PAUP*. The best model, selected by the Akaike Information Criterion (AIC), was the TIM + I + G (freq. A = 0.2902; freq. C = 0.2840; freq. G = 0.1159; freq. T = 0.3099; proportion of invariable sites = 0.7146; gamma distribution shape parameter = 0.9549). The robustness of the branching pattern of the trees was tested by 10 000 bootstrap replicates for MP, ML and NJ analyses. All the above analyses were also performed for the CR and the cyt *b* separately, with models optimized for each dataset. Nucleotide diversity (π) of each clade was calculated using ARLEQUIN version 2.0 (Schneider *et al*. 2000). Results were compared using an ANOVA.

Results

Data analysis

A total of 45 different haplotypes from 80 samples of *Vipera berus* were obtained for the CR. There were 75 (8.1%) variable sites (10.9% including outgroup), of which 45 (4.9%, 5.3% including outgroup) were phylogenetically informative under MP criteria. Four indels occurred within the *V. berus* sequences, with another three indels when including the outgroup. For the CR and cyt *b* combined analysis, comprising 1961 bp from 50 samples of *V. berus*, 147 sites (7.5%) were variable among the ingroup taxa (11.0% including outgroup), with 91 sites (4.6%) being parsimony informative (4.8% including outgroup). Uncorrected (p) distance divergence ranged from 0% to 2.6% within *V. berus*, and from 4.8% to 5.7% between *V. berus* and *Vipera seoanei*.

We tested for substitution saturation in the CR and in the cyt *b* (whole gene and each codon position separately). Within *V. berus*, signs of saturation were present only for transitions in the cyt *b* at third codon position among ingroup taxa, and became evident only among *V. berus* and the outgroup in both datasets (plots not shown). Therefore, saturation was not considered to be a significant factor and all nucleotide positions were used in subsequent analyses. Moreover, the intraspecific uncorrected pairwise sequence divergences of the two mtDNA data sets (CR and cyt *b*) indicated a similar rate of substitution ($y = 1.076x$, $r^2 = 0.507$, $P < 0.01$).

Phylogenetic analysis

A partition homogeneity test showed no significant conflict between cyt *b* and CR data sets ($P = 0.143$). Consequently, we only present the analyses performed with the combined sequence data.

The MP as well as the NJ and the ML reconstructions revealed three major, well-supported phylogeographical clades, with the most geographically widespread clade (the Northern clade) clearly being composed of five geographically and phylogenetically distinct subclades, albeit with low bootstrap support (Fig. 2). The first clade (the Italian clade; bootstrap support of NJ, MP and ML analyses: 88/97/92) included samples from Italy, northern Slovenia, Austria (except its extreme western part) and southeastern

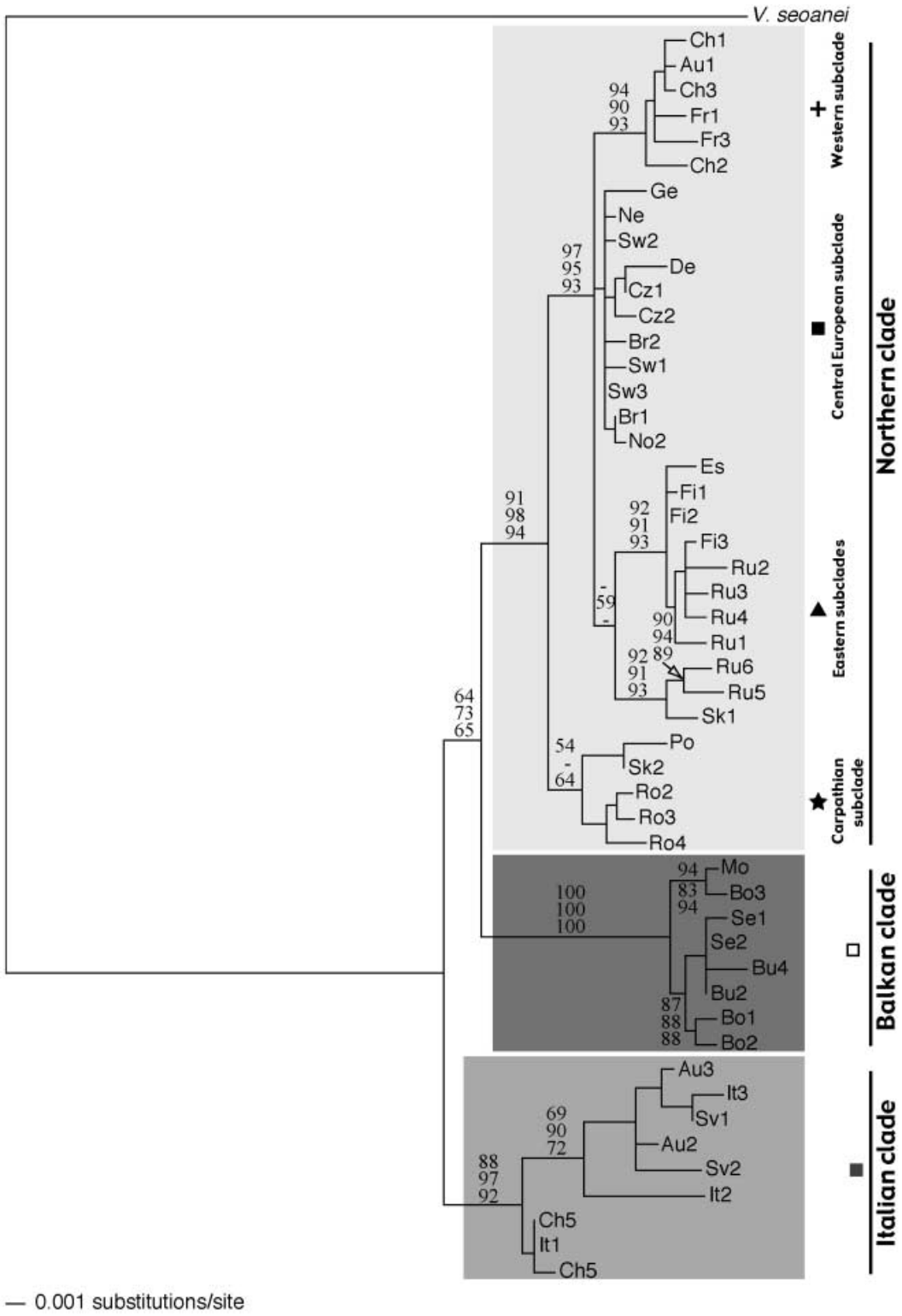

Fig. 2 Maximum-likelihood tree from combined data (mtDNA control region and cytochrome *b* sequences, totalling 1961 bp) for *Vipera berus*. Values of bootstrap support are shown for nodes found in more than 50% of 10 000 trees for neighbour joining (top), maximum-parsimony (middle) and likelihood analyses (bottom). Individual ID corresponds to population code in Fig. 1 and Appendix. Note that two individuals were analysed from the same locality, Ch5.

Switzerland. The second clade (the Balkan clade; bootstrap support: 100/100/100) contained all *Vipera berus bosniensis* samples from the Balkan Peninsula. The third clade (the Northern clade; bootstrap support: 91/98/94) included all adders from Europe east, north and west of the Alps as well as all animals from the Asian part of the distribution range. This clade can be subdivided into a basal Carpathian subclade, including adders from Romania, eastern Slovakia and southern Poland and the remaining haplotypes, which form a separate northern group with high bootstrap support, which in turn contains four monophyletic subclades. All adders from Russia, Finland, Estonia and northern Slovakia formed two subclades (the Eastern subclades, which did not yield monophyletic support to the subspecies *Vipera berus sachalinensis*), as did adders from France, Switzerland and the western extreme of Austria (the Western subclade). All other haplotypes within the Northern clade formed a group based on a single unambiguous character, thus lacking bootstrap support. Most haplotypes within this group (Central European subclade) were phylogenetically unresolved, with variation only at terminal branches, but all originate from Northwestern Europe.

Genetic diversity

Nucleotide diversity was lowest within the Balkan clade (0.32% ± 0.20), whereas both Italian (0.63 ± 0.35%) and Northern (0.61 ± 0.32%) clades showed comparable values. The difference between the Northern clade and the southern clades (Balkan and Italian clades) was not significant (ANOVA: $F = 0.221$, $P = 0.72$). Corrected divergences (using the model defined by MODELTEST) between clades ranged from 1.7% to 2.0%, with the Italian and the Northern clades being the least divergent, whereas the largest distance was observed between the Balkan and the Italian clades. Within the Northern clade, the Carpathian subclade showed the highest genetic diversity (0.45%), whereas the Central European subclade had the lowest values (0.17%). However, no significant differences between the five subclades were detected (ANOVA: $F = 2.11$, $P = 0.11$).

Discussion

Comparison of the substitution rate between CR and cyt b

The CR has often been used for analyses of intraspecific phylogeography because of a high variability due to a putatively lower level of functional constraints in comparison with mitochondrial coding regions (e.g. Avise 2004). However, in some bird and snake species, the CR substitution rate appears to be similar or lower compared to the cyt *b* or other mitochondrial genes (Questiau *et al*. 1998; Burbrink *et al*. 2000; Crochet & Desmarais 2000; Ashton & de Queiroz 2001; Randi *et al*. 2001; Ursenbacher *et al*. 2006). Our results show that the rate of evolution of the CR in taxa of the *Vipera berus* complex is not higher than that of the cyt *b* gene, and that both sequences appear useful as molecular markers to infer intraspecific phylogeographies within the genus *Vipera*. The low evolutionary rate of the CR in some bird and snake taxa suggests a possible mutational constraint on this mtDNA region. The occurrence of two or more CR copies in some birds and in advanced snakes might have an influence on the substitution rate. The duplicated sequences have been shown to undergo concerted evolution, which may act to slow down overall substitution rate (Kumazawa *et al*. 1996; Eberhard *et al*. 2001).

Structure across the whole distribution area

The phylogenetic analysis using MP, NJ and ML approaches revealed the presence of three main clades with non-overlapping geographical distributions. The first clade is geographically restricted to Italy and surrounding regions (Austria, Slovenia and southeastern Switzerland). The second clade is located in the Balkans and comprises all samples collected from within the distribution range of *Vipera berus bosniensis*. Finally, the third clade includes adders from Great Britain in the west to far eastern Russia, and north beyond the Arctic Circle in Fennoscandia (Fig. 2). Based on geological events (the final emergence of the Isthmus of Panama), Wüster *et al*. (2002) suggested a divergence rate of 1.4%/million years (Myr) [95% confidence interval (CI): 1.09–1.77%] for cyt *b* within Viperidae. Applying this divergence rate estimate to our cyt *b* data set would date the split of the three clades to about 1.4 millions years ago (Ma) (95% CI: 1.1–1.7 Ma) (corrected mean distance between the three clades: 1.89%). Although this dating must be taken with extreme caution due to the lack of calibration of the substitution rate in *V. berus* and to the sensible overestimation of timing recent events induced by the time-dependency of molecular rates (see Ho & Larson 2006), it provides an approximate time frame. We therefore suggest that the divisions into three major clades probably occurred during the lower Pleistocene. Through this period, the species should have occupied the Balkan Peninsula, Italy and an undetermined region in the East (at or near the Carpathian Mountains). The presence in Spain of the sister taxon *Vipera seoanei*, which lives in similar environments (Kalyabina-Hauf *et al*. 2004), may have prevented *V. berus* from taking refuge in the Iberian Peninsula.

Sub-structure of the Northern clade

In addition to the phylogeographical structure outlined above, the Northern clade shows a geographically correlated inner structure. The phylogenetic analyses

suggest that this clade is divided into five geographically separated subclades (Fig. 2). Some of the nodes defining these subclades have low bootstrap support due to the few mutational differences in the sequence data. Monophyly of these clades was retained, however, in each of the NJ and ML analyses. The Northern clade contains a basal subclade located around the Carpathian Mountains (Carpathian subclade). Our present findings suggest that this region might be the source area for the Northern clade, in accordance with the data of Jaarola & Searle (2002) on field voles (*Microtus agrestis*) and Babik *et al*. (2004) on the moor frog (*Rana arvalis*). Additional regional subclades have been identified: the Eastern subclades (see Fig. 2), including all adders from Eastern Europe to Pacific Russia, comprise animals from possibly several refugia to the northeast or east of the Carpathians during the last glacial cycles [0.94% mean of pairwise divergence with other subclades; 0.7 Ma (95% CI: 0.5–0.9)]. We can assume that there was a refugial population west of the Alps (Western subclade), presumably in Central France [0.29% divergence with other subclades; 0.21 Ma (95% CI: 0.16–0.27)]. This crude divergence time estimate is concordant with a population subdivision starting at the onset of the last glaciation (Weichsel), or possibly the second to last glaciation (Saahle) (Rohling *et al*. 1998). A refugial area in south-central France has also been suggested by Guillaume *et al*. (2000) for the lizard *Lacerta* (*Zootoca*) *vivipara*, which presently is sympatric with *V. berus* in most of its distribution range, for *Vipera aspis* (Ursenbacher *et al*. 2006) and for several plant species (Konnert & Bergmann 1995; Comes & Abbott 1998; Vogel *et al*. 1999). In addition, other taxa such as *Barbus barbus* (Kotlik & Berrebi 2001), *Candidula unifasciata* (Pfenninger & Posada 2002), *Corylus avellana* (Palme & Vendramin 2002) and the genus *Asplenium* (Vogel *et al*. 1999) also provide evidence that southern France has acted as a refugial area. The Central European subclade has a low support in the phylogenetic analyses, but is defined by a single substitution shared by all members of the subclade. The split of this clade with the Eastern and the Western subclades was sufficiently old (0.40 Myr, 95% CI: 0.32–0.52) to assume that it had originated from one or several refugial areas located somewhere north of the Alps and between France and the Carpathians. Unfortunately, our sampling is not sufficient to point out more precisely the probable location of these putative refugia. Nevertheless, the description in Hungary of the northernmost European forest vegetation during the Last Glacial Maximum (LGM) (Damblon 1997; Willis *et al*. 2000; Ravazzi 2002) suggests a plausible suitable location of a refugial zone for *V. berus* during the glaciations. Moreover, phylogeographical analyses of several small mammals (Jaarola & Searle 2002; Brunhoff *et al*. 2003) as well as amphibians and insects (Schmitt & Seitz 2001; Babik *et al*. 2004; Goropashnaya *et al*. 2004; Palo *et al*. 2004; Snell *et al*. 2005) confirm that this region has harboured a temperate fauna and flora during the LGM. However, evidence of other northern refugia for cold-temperate species was reported by Haase *et al*. (2003) for *Arianta arbustorum*, by Palme *et al*. (2003) for *Betula pendula*, by Tyler (2002) for *Carex digitata* and by Rendell & Ennos (2002) for *Calluna vulgaris*. These refugia could also have harboured *V. berus*, given that all these species show comparable present-day distributions.

Recolonization routes

The populations of *Vipera berus* in Europe, corresponding to the three main clades, were probably separated into three geographically isolated groups sometime in the lower Pleistocene. Whether the separation occurred during a warm or a cold period is hard to deduce from our data. Considering the hypothesis of a split during a cold period, the likely refugia would be suitable lowlands plains in the Balkans, the Northern Italian Peninsula and the Carpathian region. However, this first split could also have occurred during a warm period of the Pleistocene. Indeed, the adder requires a low mean temperature and could have retreated to higher altitudes, such as the Balkan Mountains, the Alps and the Carpathian Mountains, possibly under competition pressure with other snakes of the genus *Vipera* more adapted to warm temperatures (like *V. aspis* and perhaps *Vipera ammodytes*). Competition between *V. berus* and *V. aspis* has been demonstrated by Monney (1996) to result in the exclusion of the former species. Thus, as most likely candidate refugial areas for both alternative hypotheses coincide (Balkans, the Northern Italian Peninsula and the Carpathian region), there is presently no way of resolving the issue.

After the first isolation events, the adder should have recolonized the European continent from these three refugia during the Lower-Mid Pleistocene (see Fig. 3a), the animals of the Northern clade occupied the majority of the distribution area of the species, whereas adders from the Balkan and the Italian clades fluctuated only slightly in distribution range, presumably with larger and more widespread lowland populations during glacial times and with refugial mountain populations during interglacials, i.e. as in the present time. The position of the Carpathian clade in the phylogenetic tree, as well as the fact that the deepest divergence within the Northern clade was detected between the Carpathian subclade and the rest of the Northern clade suggests that the recolonization of Europe probably occurred from the Carpathian region.

During Mid-Late Pleistocene, further population subdivisions occurred within the three respective main clades (especially in the Northern clade; Fig. 3b). Unfortunately, the low number of samples in the Italian and the Balkan clades is insufficient to reveal any internal structure, although a west-east structure in the Italian clade (along

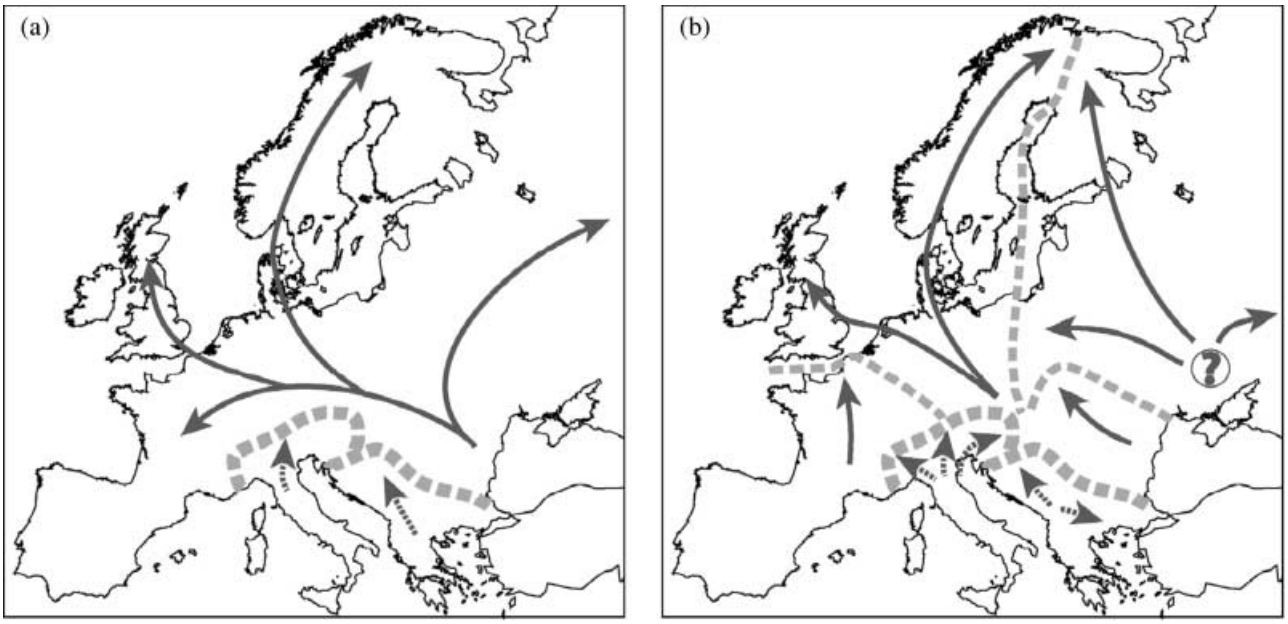

Fig. 3 Putative recolonization routes used by *Vipera berus*: (a) during the first phase of colonization of Europe (about 1 Myr); (b) during the second phase of recolonization of Europe (last glaciations). Dashed lines represent probable contact zone between clades for (a) and subclades for (b) and dashed arrows symbolize altitudinal migrations.

the Alps) and a northwest–southeast subdivision in the Balkan clade can be discerned. Within the Northern clade, the split between all subclades (at 0.2–0.8 Ma) most likely predates last glaciation. Therefore, this structuring cannot be only attributed to isolation by distance because some subclades were isolated by ice sheets or permafrost regions during the LGM. Consequently, the subdivision within the Northern clade is, arguably, due to isolated refugia used during the last glaciations, located in the Carpathians region (Carpathian subclade), somewhere east of the Carpathians (Eastern subclades), in southern France (western subclade) and in a location between Great Britain and Poland (Central European subclade). This subclade was possibly situated in or just south of the Tatra Mountains (i.e. Slovakia or Hungary). Whereas the Northern clade has experienced splitting and isolation episodes during glacial maxima of the last glaciations, southern clades (Italian and Balkan clades) have probably become subdivided during warmer interglacial periods in isolated mountain massifs. Indeed, in southern Europe, adders were probably present in lowland areas during glacial maxima (as attested for example by the occurrence of populations in the Po Plain in Italy until 100 years ago, see Scali & Gentilli 1999), allowing populations to be in contact.

The colonization paradigms suggested by Taberlet *et al*. (1998) and Hewitt (1999, 2004) showed the predominance of three main pathways of postglacial recolonization in Europe. Many temperate species studied have been found to follow these three recolonization routes and to have used Mediterranean peninsulas as refugia. Nevertheless, several temperate and cold-temperate species (Bilton *et al*. 1998; Jaarola & Searle 2002; Rendell & Ennos 2002; Tyler 2002; Brunhoff *et al*. 2003; Haase *et al*. 2003; Palme *et al*. 2003) have shown different patterns of recolonization and refugia. Some studies have recently established that other regions than Mediterranean peninsulas harboured remnant populations during LGM as the Hungarian and Carpathian regions (e.g. Willis & van Andel 2004). Our results suggest that adders used one of the routes similar to those described for some species (colonization of Europe from Carpathian or more eastern regions) during a first colonization step, then they probably followed more complex routes during the second stage of recolonization from secondary northern glacial refugia like Hungary, Carpathians or areas close to the ice sheets.

Overall, increasing evidence from climatological, palaeontological and biological data, as well as better-resolved phylogeographies, suggest a complex pattern of glacial refugia and recolonization dynamics for European biota, including the co-existence of northern and southern refugia. Clearly, range shifts during unfavourable glacial periods, recolonization routes and the location of temperate refugia varied among species with different dispersal abilities and ecological requirements. We believe that more phylogeographical studies of cold tolerant species with large distribution areas should be undertaken in order to refine the understanding of recolonization routes and the locations of glacial refugia. Finally, multidisciplinary approaches

that integrate genetics with climatic model simulations, palynology and biogeography should provide further insights into the effects of past climatic fluctuations on biological diversity.

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Sylvain Ursenbacher has research interests in reptile population genetics and biogeography, and their implications in conservation. Martin Carlsson is currently pursuing research on several population genetic topics and shared an interest with the late Håkan Tegelström in studying the biogeography of Europe. Véronique Helfer is a PhD student studying population structure and phylogeography of salamanders, and is interested in amphibian and reptile population biology. Luca Fumagalli is responsible for the Laboratory for Conservation Biology at Lausanne University, with main research interests focusing on the conservation genetics and molecular ecology of many different animal species (www.unil.ch/lbc).

Appendix

Samples of *Vipera berus* used in the present study. Population code (corresponding to Figs 1 and 2), locality and collector/collection data and haplotype designation for the mitochondrial control region and the cytochrome *b* (*n* = number of individuals from which control region sequences were obtained) are presented

Taxon	Population code	*n*	Locality	Voucher number or collector	CR	Cyt *b*
V. b. berus	Sv1	1	Krma, Slovenia	ML 3501	H30	J36
	Sv2	1	Jelendol, Slovenia	ML 5460	H32	J38
	Au1	1	Vorarlberg, Austria	D. Huber	H2	J3
	Au2	1	Zederhaus, Austria	DEE-LBC collection	H33	J39
	Au3	1	Puchberg a. Schneeberg, Austria	DEE-LBC collection	H34	J40
	It1	1	Pontebba, Italy	MCSNLU V 1228	H28	J33
	It2	1	Val Cimoliana, Italy	MCSNF RE0209	H31	J37
	It3	1	Ugorizza, Italy	Y. Surget	H30	J35
	Ch1	6	Vallée de Joux Switzerland	DEE-LBC collection	H1	J1
	Ch2	5	Préalpes fribourgoises, Switzerland	DEE-LBC collection	H3	J4
	Ch3	4	Uri, Switzerland	DEE-LBC collection	H4	J5
	Ch4	1	Sils, Switzerland	DEE-LBC collection	H28	
	Ch5	5	Pontresina, Switzerland	DEE-LBC collection	H28, H29	J32, J34
	Fr1	1	Massif Central, France	G. Thierry	H1	J2
	Fr2	1	Massif Central, France	G. Thierry	H1	
	Fr3	1	Paimpont, France	B. Heulin	H5	J6
	Fr4	1	Ardennes, France	G. Thierry	H5	
	Ne	1	Putten, Netherlands	ZMA 12133, Amsterdam	H8	J9
	Br1	1	Bangor, Wales	R. Thorpe	H6	J7
	Br2	1	Wales	R. Jones	H7	J8
	Ge	1	Fulda, Germany	U. Joger	H9	J13
	Cz1	1	Hranicna, Czech Republic	D. Modry	H10	J14
	Cz2	1	Hradec Kralove, Czech Republic	ML 3675	H8	J10
	Sk1	1	Skalité, Slovakia	D. Modry	H15	J18
	Sk2	1	Vihorlat Montain, Slovakia	Y. Surget	H24	J28
	Po	1	Ustrzyki Dolne, Poland	R. Maslak	H23	J27
	Ro1	2	Apuseni Montain, Romania	I. Ghira	H25	
	Ro2	1	Oradea, Romania	I. Ghira	H25	J29
	Ro3	1	Chirui, Romania	L. Krecsak	H26	J30
	Ro4	1	C'rta, Romania	L. Krecsak	H27	J31
	De	1	Fyns Hoved, Denmark	EBC collection	H11	J15
	No1	1	Kvannes, Norway	EBC collection	H8	
	No2	1	Ertvågøya, Norway	Hans Olsvik	H12	J16
	Sw1	1	Grebbestad, Sweden	EBC collection	H8	J11
	Sw2	1	Mora, Sweden	EBC collection	H13	J17
	Sw3	1	Långalma, Sweden	EBC collection	H8	J12
	Sw4	1	Ligga, Sweden	EBC collection	H8	
	Sw5	1	Staffans, Sweden	EBC collection	H14	
	Fi1	1	Karhujärvi, Finland	EBC collection	H16	J20
	Fi2	1	Hämeenlinna, Finland	EBC collection	H16	J21
	Fi3	1	Polvijärvi, Finland	S. Paasanen	H17	J22
	Fi4	1	Övermark, Finland	EBC collection	H16	
	Fi5	1	Oulunsalo, Finland	EBC collection	H18	
	Es	1	Hiumaa, Estonia	P. Ernits	H16	J19
	Ru1	2	Shulgino, St Petersburg Prov, Russia	ZISP 20862#21	H16, H19	J23
	Ru2	2	Dedilovskie Vysetki, Tula Prov., Russia	ZISP 21098#87/06177	H16, H20	J24
	Ru3	2	Altai, Russia	N. Ananjeva	H17, H21	J25
	Ru4	1	Krasnoyarski Kray, Russia	ZISP18310, St Petersburg	H22	J26

Appendix *Continued*

Taxon	Population code	*n*	Locality	Voucher number or collector	CR	Cyt *b*
V. b. bosniensis	Bo1	1	Bihac, Bosnia	ML 3622	H36	J42
	Bo2	1	Slavonski Brod, Bosnia	ML 3621	H37	J43
	Bo3	1	Treskavica, Bosnia	ML 3882	H35	J41
	Mo	1	Bjelasica mountain, Montenegro	L. Tomovic & J. Crnobrnja-Isailovic	H38	J44
	Se1	1	Kopaonik mountain, Serbia	L. Tomovic & J. Crnobrnja-Isailovic	H39	J45
	Se2	1	Stara Planina mt., Serbia	L. Tomovic & J. Crnobrnja-Isailovic	H40	J46
	Bu1	1	Vitoshe Mountain, Bulgaria	A. Westerström	H41	
	Bu2	1	Pirin Mountain, Bulgaria	A. Westerström	H43	J48
	Bu3	1	Stara Planina, Bulgaria	A. Westerström	H43	
	Bu4	1	Musala Mountain, Bulgaria	A. Westerström	H42	J47
V. b. sachalinensis	Ru5	1	Khabarovsky Kray, Russia	ZISP 18635, St Petersburg	H44	J49
	Ru6	1	Yuzhno-Sakhalinsk, Russia	ZISP 20282, St Petersburg	H45	J50
V. seoanei		1	Spain	T. Durand	outgroup	

Museum and Institute acronyms: ML, Prirodosloveni Muzej Slovenije, Ljubljana (Slovenia); DEE-LBC, Département d'Ecologie et Evolution — Laboratoire de Biologie de la Conservation, Lausanne (Switzerland); MCSNLU, Museo Cantonale di Storia Naturale, Lugano (Switzerland); MCSNF, Museo Civico di Storia Naturale di Ferrara (Italy); ZMA, Zoological Museum of Amsterdam (Netherlands); EBC, Department of Conservation Biology and Genetics, Evolutionary Biology Centre, Uppsala (Sweden); ZISP, Zoological Institute of St Petersburg (Russia).