ORIGINAL ARTICLE

Phylogeography of the *Vipera ursinii* complex (Viperidae): mitochondrial markers reveal an east–west disjunction in the Palaearctic region

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ABSTRACT

Aim The aim of this study was to elucidate the phylogeographical pattern of taxa composing the *Vipera ursinii* complex, for which the taxonomic status and the dating of splitting events have been the subject of much debate. The objectives were to delimit potential refugia and to date splitting events in order to suggest a scenario that explains the diversification of this species complex.

Location Western Europe to Central Asia.

Methods Sequences of the mitochondrial cytochrome *b* and NADH dehydrogenase subunit 4 (*ND4*) genes were analysed for 125 individuals from 46 locations throughout the distribution range of the complex. The phylogeographical structure was investigated using Bayesian and maximum likelihood methods. Molecular dating was performed using three calibration points to estimate the timing of diversification.

Results Eighty-nine haplotypes were observed from the concatenation of the two genes. Phylogenetic inferences supported two main groups, referred to in this study as the 'ursinii clade' and the 'renardi clade', within which several subclades were identified. Samples from Greece (*Vipera ursinii graeca*) represented the first split within the *V. ursinii* complex. In addition, three main periods of diversification were revealed, mainly during the Pleistocene (2.4–2.0 Ma, 1.4 Ma and 1.0–0.6 Ma).

Main conclusions The present distribution of the *V. ursinii* complex seems to have been shaped by Quaternary climatic fluctuations, and the Balkan, Caucasus and Carpathian regions are identified in this study as probable refugia. Our results support a south–north pattern of colonization, in contrast to the north–south colonization previously proposed for this complex. The biogeographical history of the *V. ursinii* complex corroborates other biogeographical studies that have revealed an east–west disjunction (situated near the Black Sea) within a species complex distributed throughout the Palaearctic region.

Keywords

***Acridophaga*, alpine species, Bayesian inference, European vipers, maximum likelihood, mitochondrial markers, molecular dating, Quaternary climatic fluctuations, steppe, *Vipera ursinii* complex.**

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INTRODUCTION

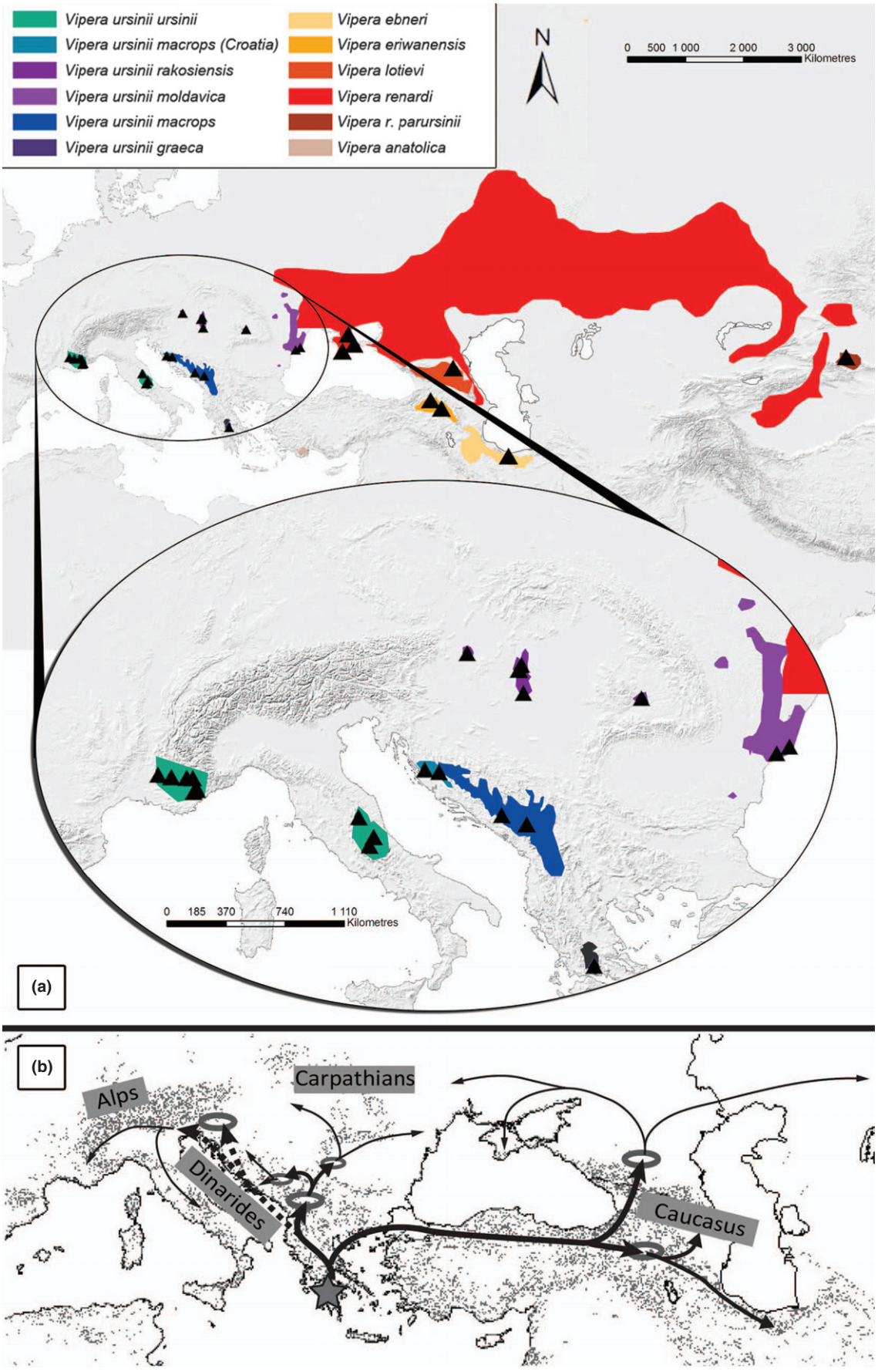
In Europe, most temperate species survived the Quaternary glaciations in refugia located in southern Europe, mainly in the Iberian, Italian and Balkan peninsulas (Taberlet *et al.*, 1998; Hewitt, 2000; but see also Provan & Bennett, 2008). In addition to these southern zones, more northerly refugia have been identified for some cold-tolerant species (Bhagwat & Willis, 2008). In each glacial period, populations diverged through selection, genetic drift and local adaptation within isolated refugia. During subsequent warmer interglacial periods (such as the one we are currently experiencing), a rapid northward re-colonization occurred. Repeated cycles of population expansion and contraction have left traces in the gene pools of current populations that can be used for historical reconstruction (Avise, 2000).

Several phylogeographical studies have focused on European *Vipera* species. The biogeographical history of *Vipera ammodytes* reveals a pattern of colonization pre-dating Pleistocene climatic fluctuations (during the Early Pliocene) and of high genetic diversity within the Balkan Peninsula (Ursenbacher *et al.*, 2008). Phylogeographical analysis of *Vipera aspis* suggested the occurrence of multiple Mediterranean refugia, but molecular dating was not available at the time of this analysis (Ursenbacher *et al.*, 2006a). Furthermore, based on morphological differentiation, Brito *et al.* (2008) proposed several population refugia of *Vipera latastei* in the Iberian Peninsula resulting from Pleistocene glaciations. In addition to the Italian and Balkan peninsulas, more northerly refugia (from France to Russia) have been identified for the adder (*Vipera berus*), which differentiated into three major clades during the Pleistocene (Ursenbacher *et al.*, 2006b). From these studies, it can be concluded that several *Vipera* species experienced range contractions into southern peninsulas (Iberian, Balkan or Italian) during the glacial periods of the Pleistocene, whereas *V. berus* persisted in more northerly locations, consistent with it being a more cold-tolerant species.

Meadow and steppe vipers include several species and subspecies that are usually grouped in the *Vipera ursinii* complex, also referred to as the subgenus *Acridophaga* (Nilson & Andrén, 2001). Specific and subspecific divisions of the *V. ursinii* complex have been the subject of much debate among taxonomists (Eiselt & Baran, 1970; Joger *et al.*, 1992; Höggren *et al.*, 1993; Nilson *et al.*, 1995; Nilson & Andrén, 2001; Mallow *et al.*, 2003). Until 1993, all taxa were grouped within a single taxon, *V. ursinii*, but with different subspecies. Nilson *et al.* (1995) raised *V. u. eriwanensis* to the species level, and described *V. lotievi* (see Fig. 1a for its distribution). In 1999, an electrophoretic study of allozymes supported the species status of *V. renardi* (Kotenko *et al.*, 1999). In 2001, based on morphological and immunological studies, *V. u. renardi*, *V. u. anatolica* and *V. u. ebneri* were elevated to the rank of species (Nilson & Andrén, 2001). These taxa are distributed from Western Europe to Central Asia, but show highly fragmented distributions, particularly on the southern and western margins of the range (Fig. 1a). These taxa are often restricted to subalpine meadows, xerophytic montane meadows or other grasslands located on mountain ridges, and they inhabit steppes in eastern European lowlands. The global picture (see Fig. 1a) thus comprises one group of widespread lowland taxa, including *V. u. rakosiensis*, *V. u. moldavica* and *V. renardi*, and three assemblages of mountain taxa: (1) a European group (*V. u. ursinii*, *V. u. macrops*, *V. u. graeca*), (2) a trans-Caucasian group (*V. lotievi*, *V. eriwanensis*, *V. ebneri* and *V. anatolica*), and (3) a trans-Caspian–Chinese group (*V. renardi parursinii* and *V. r. tienshanica*). According to Nilson & Andrén (2001), the *V. ursinii* complex has an Asiatic origin and the lowland taxa represent a plesiomorphic lineage, leading to a scenario of colonization from continuous northern and eastern lowland populations to isolated southern mountainous areas. Moreover, as most taxa of the *V. ursinii* complex inhabit open grassland habitats in montane elevations, the question arose whether *V. ursinii* could be considered an alpine and/or a cold-tolerant species (like its sister species *V. berus*). If it is considered an alpine taxon, the colonization pattern would be the opposite of the traditional pattern for lowland species, with expansion during glaciations and contractions during warmer interglacial periods (e.g. Assefa *et al.*, 2007; Browne & Ferree, 2007). However, no exhaustive phylogeographical study of this group has yet been conducted in order to test these biogeographical hypotheses or to resolve questions concerning the status and phylogenetic relationships of the various taxa considered.

In this paper, we present a quasi-exhaustive sampling over the entire distribution range of the *V. ursinii* complex. The aim of our study was to provide rigorous phylogenetic reconstructions and molecular dating for this taxonomic complex based on mitochondrial DNA (mtDNA) sequences in order to infer the evolutionary history of the subgenus *Acridophaga*. Our objectives were to delimit and date potential refugia and to propose a scenario explaining the diversification of this complex. In particular, we aimed to test the north-to-south colonization hypothesis proposed by Nilson & Andrén (2001).

Figure 1 (a) Distributions of the various species and subspecies of the meadow and steppe viper *Vipera ursinii* complex (adapted from Nilson & Andrén, 2001 and Patrick & Vogel, 2010) in Europe and western Asia. Sampled localities are represented by black symbols. (b) Putative re-colonization routes of meadow and steppe vipers (*V. ursinii* complex). The three thicknesses of arrow refer to the three main periods of diversification: the thickest arrow corresponds to the colonization/splits occurring *c.* 2.4 Ma (the dotted line suggests a disappearance of the populations concerned), the intermediate arrow corresponds to 1.4 Ma, and the thinnest arrow to 1.0–0.6 Ma (see text for details).



MATERIALS AND METHODS

Taxon sampling

Our sampling covered the entire distribution range of the *V. ursinii* complex and included 125 individuals from 46 locations (Fig. 1a, and Appendix S1 in Supporting Information). Three other viper species (*V. berus*, *V. kaznakovi* and *V. seoanei*) were included as outgroups (Appendix S1). Tissue samples (parts of two ventral scales, tail, skin or blood samples) were obtained from individuals captured in the field or from ethanol-preserved animals (see Appendix S1).

DNA sequencing

Total genomic DNA was extracted from tissue samples using a QIAamp DNA Mini Kit (Qiagen, Courtaboeuf, France), following the manufacturer's instructions. Portions of the mitochondrial cytochrome *b* (cyt *b*) and NADH dehydrogenase subunit 4 (*ND4*) genes were amplified by polymerase chain reaction (PCR) and sequenced using the primers L14724Vb and H15914Vb for cyt *b* (Ursenbacher *et al.*, 2006b) and ND4 and H12763 for *ND4* (Arevalo *et al.*, 1994). PCR was performed in a 40-μL final volume, including 5 μL of DNA template, 10× PCR buffer (Qiagen), 25 mM $MgCl_2$, 0.25 mM of each dNTP, 10 μM of each primer and 1 U of *Taq* polymerase (Qiagen). Amplification conditions consisted of 34 cycles as follows: denaturation for 45 s at 94 °C, annealing for 60 s at 50 °C for cyt *b* and at 52 °C for *ND4*, and extension for 3 min at 72 °C. PCR products were sequenced by LGC Genomics (Berlin, Germany), using ABI 3730 XL and 3130 XL sequencers (Applied Biosystems, Foster City, CA).

Electropherograms were read and aligned using SEQUENCHER 4.2.2 (Gene Codes Corporation, Ann Arbor, MI, USA). Sequences were also translated into amino acids to check for possible amplification of pseudo-genes. Both forward and reverse sequences were checked by eye, and a consensus sequence was compiled with SEQUENCHER. A total of 206 sequences were obtained (104 for cyt *b* and 102 for *ND4*) and deposited in the European Molecular Biology Laboratory database with accession numbers FR727039–FR727105 and FR745953–FR745993 for cyt *b*, and accession numbers FR726956–FR727037 and FR745893–FR745912 for *ND4*.

Partitioning and phylogenetic analysis

Sequences were aligned manually using the ED program as implemented in MUST (Philippe, 1993). The two mitochondrial fragments were concatenated, and identical sequences were merged into haplotypes defined by COLLAPSE 1.2 (available from http://darwin.uvigo.es/). Uncorrected '*p*' distances between each pairwise sequence alignment were calculated using the COMPMAT program in MUST. As the choice of an appropriate model is a crucial issue in phylogenetics, six data partitioning strategies were tested: (1) a single partition (two genes concatenated); (2) two partitions (one per gene); (3) three partitions (one per codon position on the concatenated dataset); (4) three partitions (positions 1 + 2 of concatenated cyt *b* and *ND4*, and one for position 3 of each gene separately); (5) four partitions (two partitions per gene: one for positions 1 + 2, and one for position 3); and (6) six partitions (one per codon position for each gene separately). For each data partition, the best-fit model of sequence evolution was assigned using the likelihood ratio test generated using MODELGENERATOR 0.82 (Keane *et al.*, 2006).

Bayesian analyses were performed with MRBAYES 3.1.2 (Ronquist & Huelsenbeck, 2003) for each partitioning strategy. Four Metropolis-coupled Markov chain Monte Carlo (MCMC) algorithms were run, starting from random topologies. Two separate runs of five million generations (sampled every 500 generations) were conducted simultaneously, and TRACER 1.5 (Rambaut & Drummond, 2007) was used to check the convergence between the two runs and to determine the burn-in period. On this basis, the first 5000 phylogenetic trees were discarded, and the remaining 5000 trees were used to estimate posterior parameters and probability distributions. The optimal partition was then selected, based on the Bayes factor (Brandley *et al.*, 2005) as implemented in TRACER, and calculated as the difference in the harmonic means of the −ln likelihood scores (burn-in excluded) between two partitioning schemes. A value of 10 for the 2ln Bayes factor was used as a minimum threshold for selecting the more complex model (Kass & Raftery, 1995; Brandley *et al.*, 2005).

Phylogenetic trees were reconstructed using two probabilistic methods: Bayesian inference with MRBAYES, and maximum likelihood with RAxML 7.0.4 (Stamatakis, 2006). These two types of software allowed different models of sequence evolution to be applied to different data partitions. The best data partition with the best sequence evolution model was used to reconstruct phylogenetic trees with MRBAYES (two runs of five million generations, as previously described). As GTR is the only nucleotide substitution model available in RAxML, GTR + I + G was applied to all partitions. The robustness of nodes was evaluated with 1000 bootstrap replicates with RAxML, and a consensus tree was obtained using the CONSENSE module with PHYLIP 3.69 software (Felsenstein, 2005).

Molecular dating

Divergence dates were estimated with BEAST 1.5.4 (Drummond & Rambaut, 2007), based on sophisticated Bayesian methods incorporating: (1) a relaxed molecular clock allowing variations in rates of evolution among lineages; (2) multiple fossil calibration points; (3) prior modelling for a likely probability distribution and uncertainty of the node ages used for calibration. The use of realistic models that take into account sequence evolution and uncertainty in the dating of fossils (Yang & Rannala, 2006) has greatly improved molecular dating by avoiding overestimation of the age of internal nodes (Hugall *et al.*, 2007), which could result from an incomplete fossil record.

Molecular dating was performed with the same three calibration points as used by Wüster *et al.* (2008) for

estimating divergence dates within Viperidae: (1) the uplift of the Panamanian Isthmus that separated populations of *Prothidium*, dated at *c.* 3.5 Ma (Wüster *et al.*, 2002) – this point was modelled with a normal distribution with a mean of 3.5 Ma and a standard deviation of 0.51 Myr, providing a 95% confidence interval of 2.5–4.5 Ma; (2) the initial divergence of the Eurasian viper clade (comprising the *Macrovipera*, *Montivipera* and *Vipera* genera), dated at 20 Ma, as suggested by fossil evidence (Szyndlar & Rage, 1990); (3) the divergence between the *Sisturus* and *Crotalus* genera that occurred before 9 Ma, as suggested by a fossil vertebra of *Sisturus* (Parmley & Holman, 2007). The last two calibration points were modelled with a lognormal prior with a zero offset on 20 and 9 Ma, respectively, a default lognormal mean of 1 and a default lognormal standard deviation of 1. Divergence dates were estimated using the three calibration points simultaneously, as well as with all combinations with only two calibration points.

In order to evaluate divergence dates, the cyt *b* and *ND4* sequences of 13 additional species related to the taxa concerned by the calibration points were added to the dataset: four species of *Crotalus* (*C. adamanteus*, *C. ravus*, *C. simus* and *C. tigris*), *Macrovipera lebetina*, two species of *Montivipera* (*M. albizona* and *M. xanthina*), three species of *Porthidium* (*P. arcosae*, *P. lansbergii rozei* and *P. nasutum*), two species of *Sisturus* (*S. catenatus* and *S. miliarus*) and *Vipera ammodytes*. Three outgroups (*Natrix natrix*, *Coronella girondica* and *Naja naja*) were also included. For all taxa, the accession numbers for cyt *b* and *ND4* are given in appendix S1 of Wüster *et al.* (2008).

The best evolutionary and data partition models were selected for the timing dataset as previously described for the phylogenetic reconstruction. Three molecular clock models (strict, uncorrelated exponential and uncorrelated lognormal) were also tested, and the appropriate model was determined based on Bayes factor analysis (see above). We used a Yule branching process, which is more appropriate when considering sequences from different species (Drummond & Rambaut, 2007). Each analysis was carried out using two independent runs of 30 million generations, sampled every 500 generations, after discarding the first 10% as burn-in. We used Tracer to evaluate acceptable levels of MCMC chain mixing, the stationary likelihoods and appropriate lengths of burn-in (10%), as well as to estimate effective sample sizes for all parameters.

RESULTS

Sequence data and phylogenetic trees

The 125 *V. ursinii* individuals resulted in 89 haplotypes in the combined dataset (Appendix S1). Sequences were unambiguously aligned, and no stop codon or unusual amino acid substitutions were detected, supporting a mitochondrial origin for the sequences obtained. The total alignment constituted 1918 bp (1116 bp cyt *b* + 802 bp *ND4*).

The six partitioning strategies tested (see Materials and Methods) were evaluated based on Bayes factor analyses and using the best model selected for each partition (see results in Appendix S2a). The highest value of 2ln Bayes factor was obtained for model 4, which was composed of three partitions, treating the 1st + 2nd positions of the concatenated cyt *b* + *ND4* and each cyt *b* and *ND4* 3rd position separately. It should also be noted that the data partitioned per gene (two partitions), or per gene and position (six partitions), did not improve the log-likelihood ($-\ln L$) over treating the data as a single partition.

The Bayesian phylogenetic tree obtained with this partitioning scheme is illustrated in Fig. 2. The same partitioning strategy was also used in the partitioned maximum likelihood analysis using RAxML, but using a GTR + I + G substitution model for the three partitions. The resulting phylogenetic tree was completely congruent with the Bayesian inference, not only in its branching pattern but also in the support for the various clades (Fig. 2).

The *V. ursinii* complex, as defined in Nilson & Andrén (2001) and Garrigues *et al.* (2005), forms a monophyletic assemblage, separated from other closely related *Vipera* species (*V. kaznakovi*, *V. seoanei* and *V. berus*) with statistically strong support (Fig. 2). Contrary to morphological inference (Nilson & Andrén, 2001), the *V. ursinii* species did not appear to be monophyletic, because populations from Greece (*V. ursinii graeca*: samples H6 to H8) were basal in the *V. ursinii* complex (Fig. 2). Moreover, the mean *p*-distance between *V. u. graeca* and other subspecies of *V. ursinii* (4.5% ± 0.23; Table 1) was very similar to the genetic differentiation between *V. berus* and *V. seoanei* (4.9% ± 0.38). Among the remaining *V. ursinii* complex taxa (without *V. u. graeca*), two highly supported groups emerged (Fig. 2): one including all the *V. ursinii* subspecies except *V. u. graeca* (hereafter referred to as the 'ursinii clade'), and one containing all samples from the Crimean peninsula, the Caucasus region and regions further east (referred to as the 'renardi clade'). The ursinii clade was divided into three subclades (with maximal support, Fig. 2): an ursinii ursinii subclade, including *V. u. ursinii* from France and Italy; a Croatian subclade encompassing only the Croatian samples of *V. u. macrops* (thus indicating that *V. u. macrops* is polyphyletic); and a Balkan subclade including *V. u. rakosiensis*, *V. u. moldavica* and samples of *V. u. macrops* from Montenegro and Bosnia. The Croatian and ursinii ursinii subclades showed some affinities, but the bootstrap support was low (ML bootstrap: 73; Bayesian posterior probability: 0.69). The renardi clade was likewise composed of two subclades: an 'eriwanensis subclade' comprising *V. eriwanensis* and *V. ebneri*, and a 'renardi subclade' including *V. renardi*, *V. lotievi* and *V. parursinii*. Within all subgroups of the renardi clade, each taxon appeared to be monophyletic, except for the renardi subclade, in which *V. lotievi* was paraphyletic (Fig. 2).

Estimation of divergence times

Each of the 16 outgroup sequences represented a unique haplotype, leading to an evaluation of the divergence time based on 104 haplotypes of 1939 bp (1116 bp cyt *b* + 823 bp *ND4*). With this dataset, Bayes factor analyses (Appendix S2b)

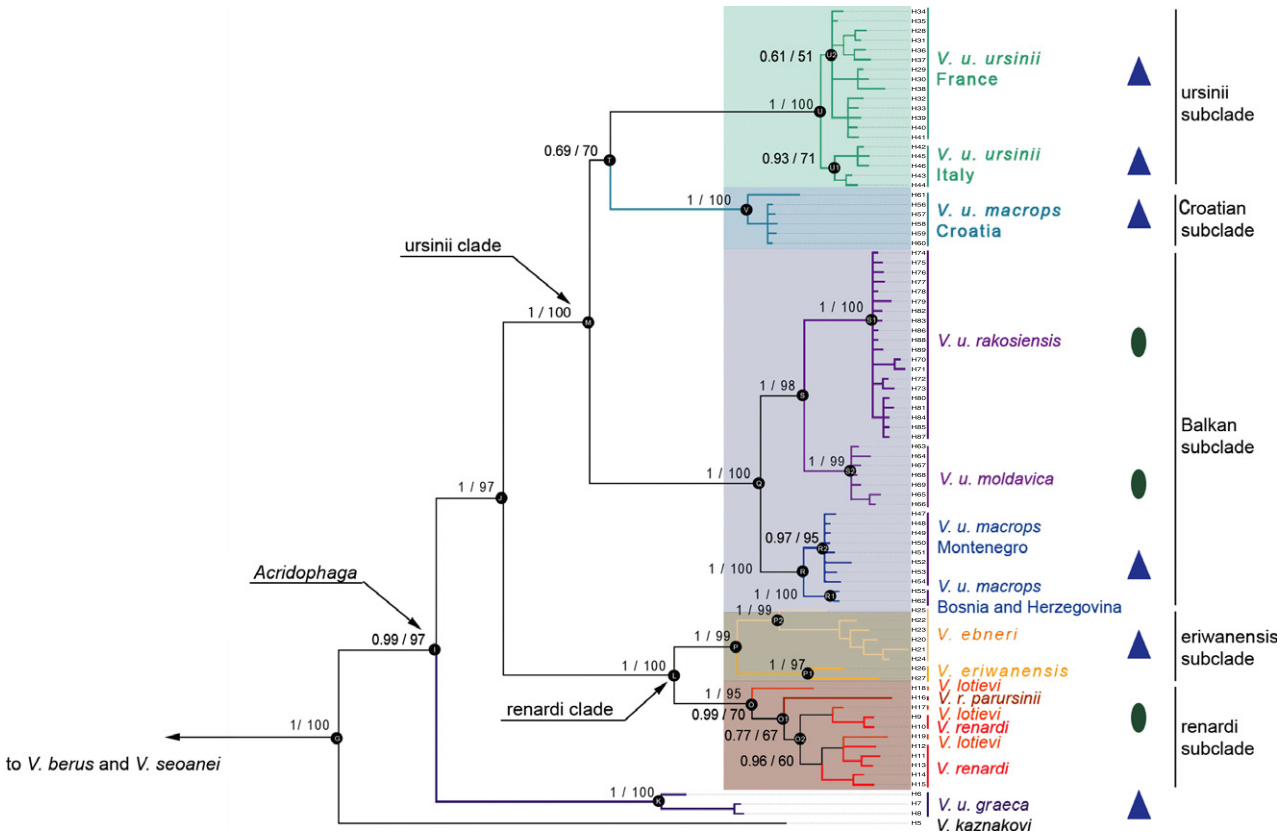


Figure 2 Bayesian phylogram between haplotypes combining the mitochondrial cyt *b* and *ND4* genes of the mtDNA of the *Vipera ursinii* complex calculated with MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) and reconstructed with FigTree 1.3.1 software. See Appendix S1 for details of the samples. Node labels correspond to nodes dated in Appendix S3. Nodal supports correspond, from left to right, to Bayesian posterior probability calculated with MrBayes and maximum likelihood bootstrap evaluated with RAxML 7.0.4 (Stamatakis, 2006). The colour code used for taxa is the same as in Figs 1(a) & 3. Habitat type is indicated for each taxon: green circles for lowland and blue triangles for mountain habitat.

Table 1 Uncorrected pairwise (*p*)-distances for concatenated cyt *b* and *ND4* within and between clades and subclades (see Fig. 2) in the *Vipera ursinii* complex in Europe and western Asia. Values are expressed as percentages, with standard deviation in parentheses.

	ursinii clade	renardi clade	ursinii subclade	Balkan subclade	renardi subclade	eriwanensis subclade	*V. (u.) graeca*	*V. kaznakovi*	*V. seoanei*	*V. berus*
ursinii clade	2.02 (1.47)									
renardi clade	4.25 (0.42)	1.58 (0.89)								
ursinii subclade	3.38 (0.55)	4.34 (0.34)	0.32 (0.15)							
Balkan subclade	2.26 (1.44)	4.43 (0.37)	3.64 (0.15)	0.82 (0.53)						
renardi subclade	4.27 (0.46)	1.76 (0.88)	4.24 (0.44)	4.49 (0.46)	0.92 (0.22)					
eriwanensis subclade	4.16 (0.39)	1.95 (0.49)	4.49 (0.40)	4.36 (0.21)	2.47 (0.38)	0.90 (0.60)				
V. (u.) graeca	4.49 (0.23)	4.63 (0.20)	4.44 (0.41)	4.47 (0.23)	4.59 (0.27)	4.68 (0.20)	0.52 (0.37)			
V. kasnakovi	5.68 (0.25)	5.40 (0.18)	5.75 (0.13)	5.81 (0.19)	5.42 (0.12)	5.35 (0.53)	4.89 (0.09)	n.a.		
V. seoanei	7.63 (0.29)	7.17 (0.22)	7.72 (0.24)	7.70 (0.43)	7.13 (0.30)	7.23 (0.10)	6.94 (0.12)	6.85 (0.00)	n.a.	
V. berus	6.00 (0.27)	5.79 (0.27)	6.26 (0.36)	5.99 (0.47)	5.72 (0.34)	5.91 (0.10)	5.71 (0.26)	5.63 (0.14)	4.99 (0.38)	1.17 (0.94)

n.a.: data not available.

revealed that the best partitioning strategy was represented by model 6, with six partitions (one per gene and per codon position). The Bayes factor indicated (2ln Bayes factor > 10) that the uncorrelated exponential relaxed clock model, without spatial autocorrelation in rates of sequence evolution, was significantly more adapted than other clocks tested to our dataset and was implemented in subsequent BEAST analyses (likelihood values for the strict, the uncorrelated exponential and the uncorrelated lognormal relaxed clock models were −14,340.936, −14,296.232 and −14,314.004, respectively).

The divergence time estimates for the 32 significantly supported nodes among *Vipera* species are provided in Appendix S3 for the four analyses run with two (each combination of two calibration points) or three constrained nodes. The four analyses provided very similar estimates for the great majority of nodes. Exceptions were observed for the oldest nodes (i.e. Eurasian vipers, node D in Fig. 3 and Appendix S3) estimated in run 2, which used the most recent calibration points (3.5 and 9 Ma). However, recent node evaluations were similar to in the other three analyses.

Divergence times estimated with the three constrained nodes, as well as the 95% highest posterior densities (HPD), are reported for each node on the chronogram in Fig. 3. The separation of *V. u. graeca* from the ursinii and renardi clades, as well as the split between *V. berus* and *V. seoanei*, was estimated to occur around 3.2–3.6 Ma (95% HPD within the *V. ursinii* complex: 2.3–5.1 Ma). In addition, three main phases of divergence could be recognized during the diversification of the *V. ursinii* complex (Fig. 3; Appendix S3). The first divergence period occurred *c.* 2.1–2.5 Ma (95% HPD: 1.1–3.9 Ma) and corresponds to divergence between the ursinii and renardi clades. The second event is estimated to have occurred *c.* 1.4 Ma (95% HDP: 0.7–2.3 Ma) and represents the origin of the Balkan, eriwanensis and renardi subclades. The two remaining subclades (ursinii ursinii and Croatian) diversified more recently, at 0.6 Ma (Figs 1b & 3; Appendix S3). Finally, most of the subclades, subspecies and species (*V. u. ursinii*, Croatian subclade, *V. u. rakosiensis*, *V. u. moldavica*, *V. u. macrops* without the Croatian samples, *V. renardi*, *V. r. parursinii*, *V. ebneri*, *V. eriwanensis* and *V. lotievi*) differentiated simultaneously, between 0.8 and 0.4 Ma (95% HPD: 0.1–1.7 Ma).

DISCUSSION

Tempo of diversification and locations of refugia

Biases in the estimated calibration points (such as uncertainty about the fossil age) can lead to erroneous time estimates. However, two factors support our molecular dating. First, congruent estimations were obtained between the different simulations with all or only two constrained nodes (Appendix S3), thus leading to a realistic assumption that these calibration points are reciprocally compatible. Second, the timing obtained for the divergence of Eurasian vipers (node D in Fig. 3: 22.8 Ma; 95% HPD: 20.1–27.0 Ma) is similar to the estimate obtained by Wüster *et al.* (2008) (20 Ma; 95% HPD: 18.6–27.1 Ma).

Considering that *V. ursinii* is adapted to grassland habitats, the ancestor of *V. ursinii* was probably widely distributed throughout Eurasia during the Pliocene period (1.8–5.0 Ma). The occurrence of numerous fossils in Central Europe attributed to the *V. berus* complex (fossils of *V. ursinii* are nested within the *V. berus* complex, the two species groups being very difficult to distinguish based only on bones; Szyndlar & Rage, 2002) supports this hypothesis. Around 4 Ma, climatic conditions became warmer and drier in Europe than present-day conditions (Zagwijn, 1985; van Dam, 2006). Steppe environments were consequently more widespread, suggesting a contraction period for alpine grassland species owing to the increase in elevation of the upper forest limit. Thus, *V. ursinii* might have found refuge in the southern Balkan Peninsula in Greece, where the climate was drier than in other places in Europe (van Dam, 2006). The first offshoot among *Acridophaga* in Greece suggests that the last common ancestor of the *V. ursinii* complex inhabited this region and that the first split within this complex occurred at the end of the Early Pliocene (*c.* 3.6 Ma). After the first split, three major phases of diversification were detected within the *V. ursinii* complex: between 2.4 and 2.0 Ma, at 1.4 Ma, and, more recently, between 1.0 and 0.6 Ma. These phases correspond, respectively, to the divergence of clades, of subclades and to diversifications within each terminal group. On the basis of geophysical parameters, Lisiecki & Raymo (2007) identified three important climatic transition phases during the Plio-Pleistocene, dated at 2.5, 1.4 and 0.8 Ma. Consequently, the clear correlation between the Plio-Pleistocene transitions and the *V. ursinii* splits suggests that Plio-Pleistocene climatic oscillations had a significant impact on the phylogeographical groups, and that the major splits occurred during warm periods.

Thus, three regions might be identified as putative spatio-temporal refugia.

1. Between 2.4 and 2.0 Ma, the ursinii and renardi clades emerged synchronously (nodes L and M in Fig. 3). The first major cooling of the Quaternary is recorded as being between 2.6 and 2.4 Ma in Israel (Horowitz, 1989) and in the Netherlands (Zagwijn, 1985). In this context, our timing suggests that the split, and consequently the isolation, of the ursinii and renardi clades occurred during a warm period (just after the most significant cooling of the Quaternary) that led to an upward shift in the tree line and thus to a range constriction to alpine habitats. The ursinii clade probably differentiated in the Central Balkans, and the renardi clade between Anatolia and the Caucasus Mountains. Afterwards (2.1 Ma), a refugium for the group, including *V. ursinii* and populations from Croatia, emerged in the North Dinarides.

2. Then, the Balkan, eriwanensis and renardi subclades (nodes O, P and Q in Fig. 3) arose simultaneously at *c.* 1.4 Ma. Here again, we can hypothesize that three additional refugia developed during a warm period, suggested by an elevation of the sea level (Krantz, 1991): one refugial area probably existed in the southern part of the Balkan Peninsula (for the Balkan subclade), and two refugial zones around the Caucasus Mountains (Fig. 1b).

3. More recently, at about 0.6 Ma, *V. u. ursinii* (French and Italian populations), *V. u. macrops* (Montenegrin and Bosnian populations) and *V. eriwanensis* diversified simultaneously (Fig. 3). Again, this corresponds to a warm period (Zagwijn, 1985).

The three major periods at which split events are observed in the *V. ursinii* complex strongly suggest range contractions to small isolated refugia during warmer interglacial periods, and expansions during glaciations. Therefore, as we are presently experiencing a warm period, it could be inferred that the

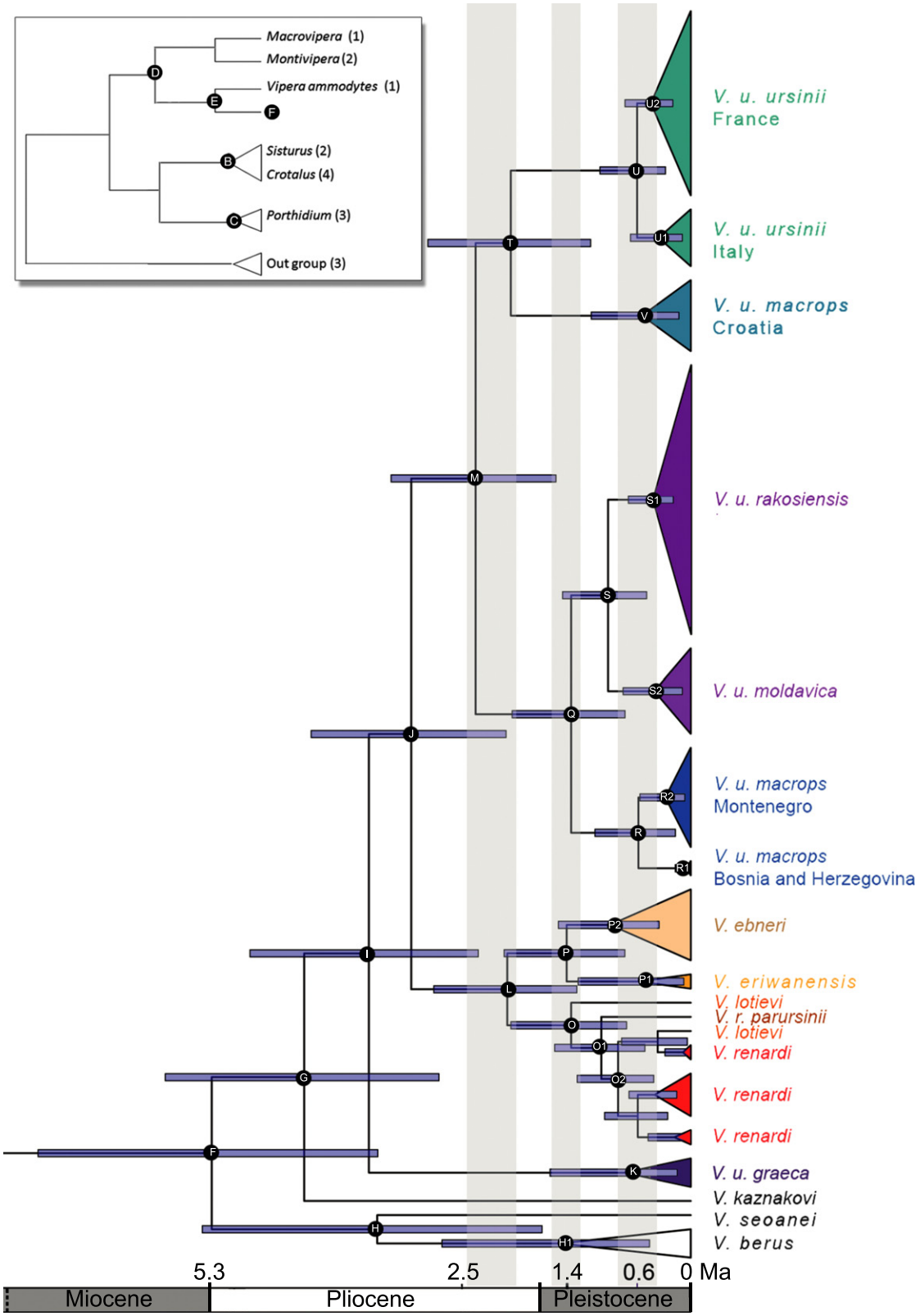


Figure 3 BEAST 1.5.4 (Drummond & Rambaut, 2007) ultrametric tree showing the timing of evolution amongst the *Vipera ursinii* complex calibrated on nodes C (3.5 Ma), B (9 Ma) and D (20 Ma). The inset tree presents the root region with the 16 additional taxa used for dating purposes (see Materials and Methods). Blue bars at nodes indicate 95% highest posterior densities. The colour code used for each taxon is the same as in Figs 1(a) & 2, and nodes are labelled as in Fig. 3. Vertical grey blocks represent the three main periods of *V. ursinii* complex diversification (see text for details).

current distribution of the *V. ursinii* taxa, at least in mountainous areas, might reflect the highly fragmented distribution within small refugia observed during warm periods. This restriction to isolated mountainous populations has important consequences for taxonomic conservation (Ferchaud *et al.*, 2011; see below).

Our study reveals that the *V. ursinii* complex presents a high degree of genetic differentiation in south-eastern Europe (six genetic clades identified). First, mitochondrial analysis grouped *V. u. rakosiensis* and *V. u. moldavica* (the two lowland taxa of *V. ursinii*) together, suggesting a common ancestor that could have survived somewhere in the Carpathian Mountains. This region has been identified as a glacial refugium for several cold-tolerant species (Jaarola & Searle, 2002; Babik *et al.*, 2004). Second, our study revealed that the sister taxon of the *rakosiensis* + *moldavica* group is represented by a well-supported group including samples from Bosnia–Herzegovina and Montenegro. This strong differentiation within the *V. ursinii* complex in the Balkan area has never been suggested from previous morphological data (Nilson & Andrén, 2001). Thus, the Balkan Peninsula appears as a centre of diversification that might be explained by the hypothesis of microrefugial zones. Owing to its large habitat heterogeneity and the presence of numerous mountains, the Balkan Peninsula (as already demonstrated for the Italian and Iberian peninsulas) can be considered as a glacial refugium containing several microrefugia (Gómez & Lunt, 2007; Crnobrnja-Isailovic, 2007), leading to a high rate of endemism, particularly for species with low dispersal abilities (Blondel *et al.*, 2010) such as vipers (this study; *V. ammodytes*: Ursenbacher *et al.*, 2008), newts (Sotiropoulos *et al.*, 2007) and Trichoptera (Previsić *et al.*, 2009).

Colonization and fragmentation scenario

The common ancestor of *Acridophaga* emerged at *c.* 3.6 Ma (node I, Fig. 3, Appendix S3) somewhere in Greece. At 3.2 Ma (node J, Fig. 3, Appendix S3), populations from Greece were isolated from the common ancestor of the ursinii and renardi clades, each experiencing subsequent subdivisions.

Structuring within the ursinii clade

The lack of a direct geographical and phylogenetic relationship between the sister clade *V. u. graeca* and the ursinii + Croatian subclade suggests a complex history of colonization, disappearance and re-colonization.

1. One group probably colonized the Dinarides from Greece up to Croatia/Slovenia (node M in Figs 3 & 1b) and subsequently France and Italy. The common ancestor of this group was dated at 2.1 Ma (node T). However, the lack of populations from this subclade between Greece and Croatia suggests a disappearance of these former *V. u. ursinii* populations between 2.1 Ma (the first colonization of Croatia) and 0.6 Ma (differentiation of *V. u. macrops* in Montenegro and Bosnia–Herzegovina; see below). We can infer that the long warm period mentioned by Gibbard and van Kolfschoten (2004, 0.137–0.018 Ma) led to the extinction of the populations between Greece and Croatia.

2. At 1.4 Ma (95% HPD 0.7–2.1 Ma), another group identified as the common ancestor of the Balkan populations emerged somewhere between Greece and Montenegro (Fig. 1b). Subsequent colonization routes to the north and north-east gave rise to the Bosnian and Montenegrin populations (0.6 Ma, 95% HPD 0.2–1.1 Ma) on the one hand, and to the lowland subspecies *V. u. rakosiensis* and *V. u. moldavica* (0.9 Ma, 95% HPD 0.5–1.5 Ma) somewhere to the south of the Carpathian Mountains on the other.

Structuring within the renardi clade

The renardi clade is distributed mostly in the northern and eastern regions of the Black Sea, but according to our study its ancestors came from the Balkan Peninsula (Fig. 1b). The common ancestor of the renardi clade was nevertheless able to reach the Anatolian Peninsula, because the Bosporus, the Sea of Marmara and the Dardanelles that presently separate the Balkan Peninsula from Anatolia only appeared during the Holocene, so the Anatolian Peninsula was directly accessible at that time from the Balkan Peninsula via a terrestrial route (Kerey *et al.*, 2004). Subsequent splits within the renardi clade took place in the Caucasus region, where two colonization paths would have been possible: (1) one route remained south of the Caucasus Mountains, which led to *V. eriwanensis* on the southern slope of this massif and to *V. ebneri* to the south of the Caspian Sea; and (2) the second route crossed the Caucasus Mountains (around 1.4 Ma), leading to the speciation of *V. lotievi* in the northern Caucasus Mountains, and subsequently to colonization north of the Black Sea (*V. renardi*) and north of the Caspian Sea to north-western China (*V. renardi* and *V. parursinii* further to the east). However, only the analysis of samples from *V. u. anatolica* would back up this scenario, for which we would expect a basal position for the renardi clade.

In conclusion, the inferred colonization routes of the *V. ursinii* complex seem very similar to those of certain other European reptiles, such as the European pond turtle (*Emys orbicularis*; Fritz *et al.*, 2007) and the green lizard (*Lacerta viridis/Lacerta bilineata*; Böhme *et al.*, 2007), and follow the classical concept of western and eastern European sister species that expand and meet in Central Europe, as suggested by Nilson & Andrén (2001). The ancestors of the *V. ursinii* complex originated in Greece and then split into a western Balkan lineage (ursinii clade) and an eastern Anatolian lineage (renardi clade). According to this hypothesis, the contact zone between *V. renardi* and *V. u. moldavica* in Moldova and possibly in south-western Ukraine can thus be interpreted as a secondary contact zone. An alternative vicariance hypothesis might be that the common ancestor of the ursinii and renardi groups was distributed continuously along the sides of the Black Sea and subsequently split into two groups (renardi and ursinii) when this habitat became unsuitable. In this way, *V. u. graeca* could have resulted from an initial vicariance

event. The vicariance hypothesis might also be compatible with the geographical distribution and sister group relationships between *V. u. moldavica* and *V. u. rakosiensis* or between the French and Italian populations of *V. u. ursinii*. On the other hand, the geographical proximity and diphyletic origin of *V. u. macrops* cannot be explained by vicariance and probably requires at least two waves of colonization. Thus, it is more likely that the disjunct pattern observed for the ursinii clade results from a complex combination of extinction, colonization and vicariance events occurring during the climatic fluctuations of the Plio-Pleistocene.

With a putative pattern of colonization from north to south, Nilson & Andrén (2001) assumed that the *V. ursinii* complex arose in steppe/grassland habitats within dry lowland areas, while several isolated populations adapted to alpine meadows during glaciations. Our results do not corroborate this hypothesis, because lowland taxa (*V. u. rakosienis*, *V. u. moldavica* and *V. renardi*) are found only in terminal positions on the phylogenetic tree. On the contrary, our study suggests that the speciation events in the *V. ursinii* complex occurred in the mountains during warm periods, and that the complex adapted independently twice to lowland habitats: once in the common ancestor of *V. u. rakosiensis* and *V. u. moldavica*, and once in the ancestor of *V. renardi*. This observation allows us to argue that *V. ursinii* is a viper adapted to grassland habitats, rather than a strictly alpine or lowland specialist, inhabiting lowlands when climatic conditions are warm and dry and support steppe vegetation, but having to find refuge in mountains when conditions at lower elevations are not favourable because of forest expansion. Thus, as a grassland species, its distribution area in the mountains reduced progressively during interglacial periods owing to the elevation of the tree line. Consequently, the isolated populations found in the mountains are more likely to be old remnants of a once wider distribution rather than populations of recent origin, as suggested by Nilson & Andrén (2001). Apart from the palaeogeographical factors and, of course, human impact, other elements could have influenced the current distribution, such as competition with other snake species. Indeed, most distributions observed for European viper species are parapatric. We can consequently assume that other taxa such as *V. aspis*, *V. berus* and *V. ammodytes* could have an impact on the present distribution of meadow and steppe vipers. For instance, it is known that competition occurs between *V. aspis* and *V. berus* (Monney, 1996), but the potential competition between *V. aspis* and *V. ursinii* appears to be low (Luiselli *et al.*, 2007).

Finally, our study led to the recognition of phylogroups different from the ones currently recognized, revealing the need for taxonomic revision and re-evaluation of some taxa (see Appendix S4 for systematic relationships and taxonomic status within the *Vipera ursinii* complex).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 List of samples and haplotypes by taxon and locality.
Appendix S2 Evolutionary models and data partition models.
Appendix S3 Dating estimations for nodes of the *Vipera ursinii* complex.
Appendix S4 Systematic relationships and taxonomic status.

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BIOSKETCH

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