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Molecular phylogeography of the nose-horned viper (*Vipera ammodytes*, Linnaeus (1758)): Evidence for high genetic diversity and multiple refugia in the Balkan peninsula

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Abstract

The nose-horned viper (*Vipera ammodytes*) occurs in a large part of the south-eastern Europe and Asia Minor. Phylogenetic relationships were reconstructed for a total of 59 specimens using sequences from three mitochondrial regions (16S and cytochrome *b* genes, and control region, totalling 2308 bp). A considerable number of clades were observed within this species, showing a large genetic diversity within the Balkan peninsula. Splitting of the basal clades was evaluated to about 4 million years ago. Genetic results are in contradiction with presently accepted taxonomy based on morphological characters: *V. a. gregorwallneri* and *V. a. ruffoi* do not display any genetic difference compared with the nominotypic subspecies (*V. a. ammodytes*), involving that these subspecies can be regarded as synonyms. High genetic divergence in the central part of the Balkan peninsula is not concordant with low morphological differentiation. Finally, the extensive genetic diversity within the Balkan peninsula and the colonisation routes are discussed.

Keywords: Mitochondrial DNA; Phylogeography; Snake; Nose-horned viper; Glacial refugia; Balkan peninsula; *Vipera ammodytes*

1. Introduction

The field of molecular phylogeography has considerably progressed with the development of PCR-based laboratory techniques and the use of animal mitochondrial DNA (mtDNA) as a custom genetic marker (Avise, 2004). Molecular data can differentiate genetic lineages with distinct evolutionary histories despite analogous morphological characters. This allows the reconstruction of the historical or phylogenetic components of population structure, such as recent radiations, bottlenecks or expansions. We know for example that several European temperate species occurred far to the south of their present distribution during Pleistocene glacial periods, and moved northward after the glaciers retreated (Hewitt, 1999, 2000; Taberlet et al., 1998). During cold periods, refugia were mainly located in the Mediterranean peninsula (Iberia, Italian and the Balkans), where populations underwent genetic differentiation before expanding northward, thus providing insights into the past distribution and recolonisation processes. In Europe, phylogeographic studies have mainly focused on mammals, birds, fishes, amphibians and invertebrates (Avise, 2000). Within reptiles however, snake

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species have been less examined and in particular only a few phylogeographic studies are available to date (Guicking et al., 2002, 2006; Kalyabina-Hauf et al., 2004; Nagy et al., 2002; Thorpe, 1984; Ursenbacher et al., 2006a,b).

The members of the Palearctic venomous snake genus *Vipera* are known in Europe since the early Miocene (Szyndlar and Rage, 1999, 2002). The first viper fossil was dated to the beginning of the Miocene, 23.8 millions years ago (Mya) (Szyndlar, 1984) and belongs to the "*Vipera aspis* complex", which includes the extant species *Vipera aspis*, *V. ammodytes* and *V. latastei* (Obst, 1983). All these three species presently inhabit mostly the southern parts of the European continent (Heckes et al., 2005; Stümpel et al., 2005; Trutnau et al., 2005). Genetic results demonstrated that *V. ammodytes* is the sister species of *V. aspis* and *V. latastei*, as well as other vipers of the genus *Vipera* (Garrigues et al., 2005). During the mid-Miocene, the scarce number of fossils discovered suggests a major regression of this group, probably due to an increase of average temperatures (Szyndlar and Rage, 1999). After this period, ancestral members of this complex were again widespread in Europe (in the late Miocene and Pliocene; Szyndlar and Rage, 1999) and vipers similar to *V. ammodytes* were present north of the Carpathians at the end of the Pliocene (Szyndlar, 1984). Nowadays, the nose-horned viper (*V. ammodytes*) is widely distributed in south-eastern Europe (from northernmost Italy and southern Austria through to the Balkan countries) and spreads eastward towards the Caucasus Mountains.

Current taxonomy of the species has only been based upon morphological characters, and remains controversial. Six different subspecies have been recognised: *V. a. ammodytes* (Linnaeus, 1758), *V. a. meridionalis* (Boulenger, 1903), *V. a. montandoni* (Boulenger, 1904), *V. a. transcaucasiana* (Boulenger, 1913), *V. a. ruffoi* (Bruno, 1968) and *V. a. gregorwallneri* (Sochurek, 1974). Ulber (1994–1997) did not recognise *V. a. ruffoi* as a valid subspecies whereas Golay et al. (1993) recognised only three subspecies, *V. a. ammodytes* (including *gregorwallneri* and *ruffoi*), *meridionalis* (including *montandoni*) and *transcaucasiana*. *V. a. transcaucasiana* has been sometimes considered as a separate species (Baran and Atatür, 1998; Nilson et al., 1999; Obst, 1983). Recently, in a comprehensive review of the species, Heckes et al. (2005) accepted four subspecies, *V. a. ammodytes*, *meridionalis*, *montandoni* and *transcaucasiana*. According to that publication (and references therein) the distribution of the nominate subspecies extends from Albania and Serbia northward to Austria and north-eastern Italy. The distribution of *V. a. meridionalis* includes Peloponnese, Cyclades, continental Greece and Macedonia while *V. a. montandoni* inhabits the northern and eastern parts of Bulgaria and eastern Romania. In contrast, Golay et al. (1993), Christov et al. (1997) and Christov and Beshkov (1999) approve to synonymise the last two subspecies. Most recently, Tomović (2006) demonstrated that there are mainly two morphologically different population groups on the southern Balkan peninsula: the more southern populations representing *V. a. meridionalis* inhabit only the Peloponnese, the Cyclades islands and central Greece, while southern Albania, northern Greece, most of Bulgaria (except the north-western part), eastern Romania, the former Yougoslavian republic of Macedonia, as well as the southernmost part of Serbia are occupied by *V. a. montandoni*.

It has been suggested that the complex geological history of the Balkan peninsula and the Hellenic area since the late Tertiary has contributed to the diversification of the terrestrial fauna (Babik et al., 2005; Beerli et al., 1996; Lymberakis et al., 2007; Poulakakis et al., 2003; Sotiropoulos et al., 2007; Wallis and Arntzen, 1989). In the Balkan peninsula, changes of relief, emergence and disappearance of orographic and hydrographical barriers were frequent (Andjelković, 1988; Oosterbroek and Arntzen, 1992; Rage and Rocek, 2003). In particular, the Hellenic region has experienced multiple events of land connection due to the fluctuation of the Mediterranean sea level. For instance, the isolation of different islands since the Tortonian (about 8 Mya) produced a high biological diversity and endemism (e.g. Fattorini, 2002; Kasapidis et al., 2005; Lymberakis et al., 2007; Poulakakis et al., 2005; Sfenthourakis, 1996; Sfenthourakis and Legakis, 2001) . However, fluctuations of the Mediterranean sea level during the Pleistocene have reconnected some Hellenic Islands to the mainland and, consequently, allowed other species to colonise these islands from the continent. Thus, genetic studies conducted on taxa from these regions can improve the understanding of the colonisation routes and past geological events.

The present study investigated the phylogeography of *V. ammodytes* across its whole distribution, using mtDNA sequences obtained from the cytochrome *b* (cyt *b*) gene, the 16S rRNA (16S) gene and the noncoding control region (CR). In particular, our aims were (i) to examine the phylogenetic relationships in *V. ammodytes* using molecular data, and compare the mtDNA phylogeny with published morphological taxonomies (ii) to date main cladogenetic events; (iii) to identify historical expansion routes and (iv) to check the influences of Pleistocene glaciations on patterns of genetic differentiation.

2. Material and methods

2.1. Sample collection and DNA extraction

Fifty-nine individuals of *V. ammodytes* including representatives of all recognised subspecies and covering most of its distribution range were included in the analyses. Localities are shown in Fig. 1 and details are given in Appendix A. One specimen of *V. aspis* was used as outgroup. *V. ammodytes* was demonstrated to be the sister species of *V. aspis* and *V. latastei* (Garrigues et al., 2005). An additional outgroup (*Macrovipera lebetina*) has also been tested

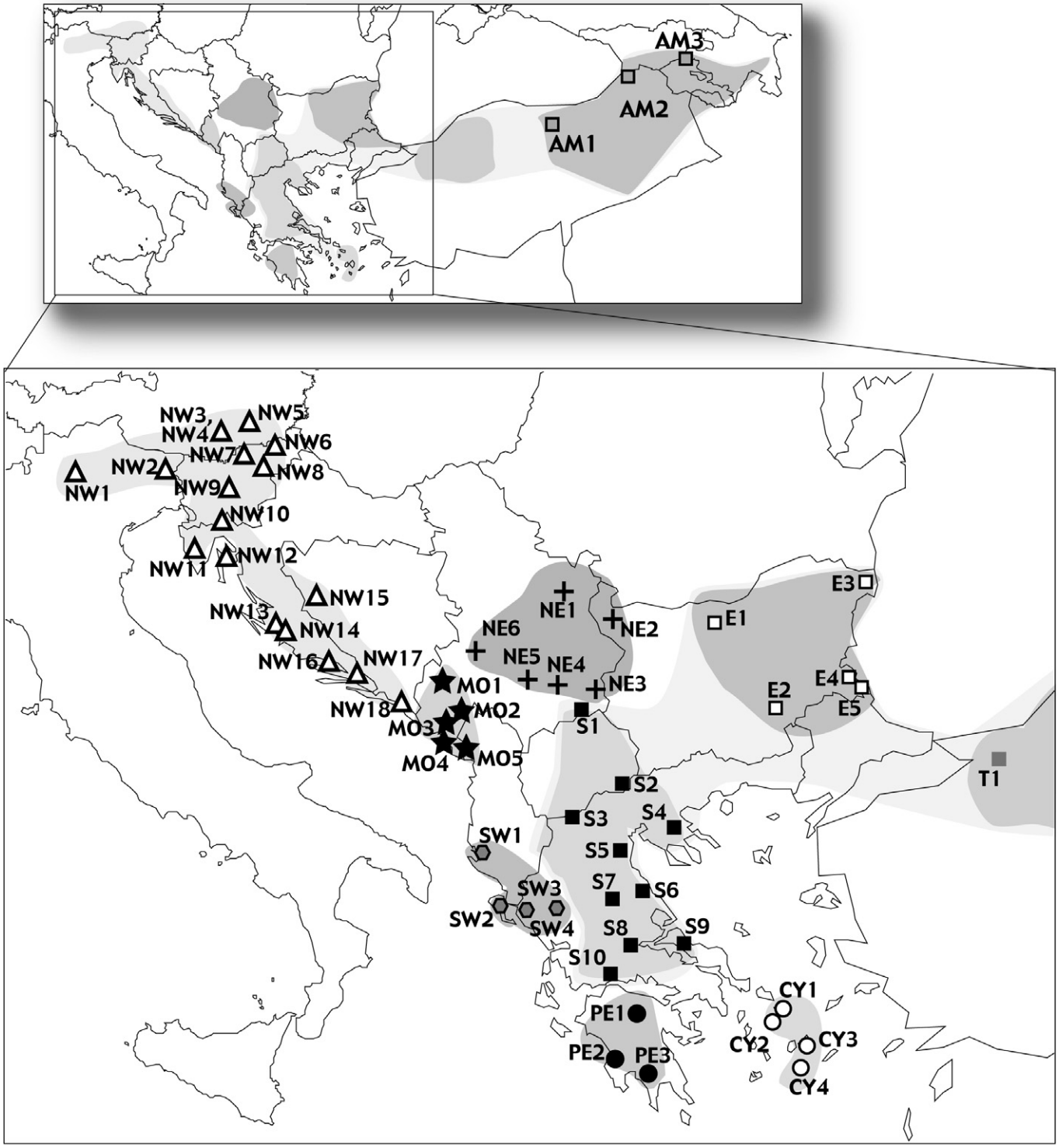

Fig. 1. Sampling localities of the 59 *Vipera ammodytes* analysed in this study. Symbols and different shades of grey correspond to genetic clades (see Fig. 2). MO corresponds to samples of the Montenegrin clade, NE of the north-eastern clade, NW of the north-western clade, SW of the south-western clade, CY of the Cyclades clade, PE to the Peloponnese clade, T of the Turkish subclade, E of the eastern subclade, S of the southern subclade and AM of the Asia Minor subclade.

and gave similar results. Total genomic DNA was extracted from ethanol preserved specimens and blood samples using standard phenol–chloroform protocol (Sambrook et al., 1989) or QIAamp DNA Mini Kit (Qiagen), respectively.

2.2. Genetic analysis

A fragment of the mtDNA cytochrome *b* (cyt *b*) gene was amplified by polymerase chain reaction (PCR) using primers LS14841 (5′-GGATCAAACATTTCAACTT-

GATG-3′) and HCYTV_L (5′-AGGCTCCAGCAACC CATTAGG-3′). A portion of the 16S rRNA (16S) gene was amplified using primers 16A1 (5′-GTATCC TAACCGTGCAAAG-3′) and H3056 (Mayer et al., 2000). For the mtDNA control region (CR), we amplified two portions separately using the two primer pairs L16148VA/H16551VA and L16571VA/H690 (Kumazawa et al., 1996; Ursenbacher et al., 2006a). All PCRs were performed 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 45 s at 50 °C for cyt *b* and 16 S, 56 °C for the first portion and 59 °C for the second portion of the CR, and extension for 60 s at 72 °C. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen). Cycle sequencing was performed with primers H16551VA, L16571VA and H690 for CR, 16A1 for 16S and LS14841 and HCYTV_L for cyt *b*, respectively, in 10 μl containing 2–5 μl of amplified DNA, 1 μl of 10 μM primer and 4 μl of ABI PRISM™ Dye Terminator 3.1 cycle sequence Ready Reaction Kit (Applied Biosystems). Water was added up to 10 μl. Reaction sequences were visualised on an ABI 3100 automated sequencer (Applied Biosystems). Sequences were deposited in GenBank (Accession Nos. DQ186081–186198 and DQ186476–DQ186520).

2.3. Phylogenetic analysis

Mitochondrial DNA sequences were aligned using ClustalX 1.83 (Thompson et al., 1997). We did not detect any indels in the 16S section examined (400 bp), therefore secondary structure differences were not considered in further analyses. The possibility of saturation for 16S and for cyt *b* at first, second and third codon positions, as well as for transitions and transversions at third codon position, was evaluated by plotting uncorrected pairwise sequence divergences against Tamura–Nei pairwise divergences (Tamura and Nei, 1993), calculated using PAUP* 4.0b10 (Swofford, 2002). To compare the relative rate of substitution between the three mtDNA regions, we plotted the uncorrected pairwise distances of CR versus cyt *b* gene, as well as 16S versus CR and 16S versus cyt *b*.

We performed a partition homogeneity test (using 1000 replicates) in order to examine whether the three analysed regions could be combined in a unique data matrix (Farris et al., 1995). To test for phylogenetic signal occurrence, skewness values of the tree length distribution (g_1 statistics, see Hillis and Huelsenbeck, 1992) were estimated from random samples of 2×10^6 trees generated by PAUP* for the combined dataset, for the CR, the 16S and the cyt *b* separately, as well as for all codon positions of the cyt *b* independently.

To determine the appropriate model of sequence evolution, the program MODELTEST 3.7 (Posada and Crandall, 1998) was used. The chosen model (using AIC procedure) was applied to the data matrix in order to produce maximum likelihood (ML) estimates using PHYML (Guindon and Gascuel, 2003). Maximum parsimony (MP) analyses were performed using PAUP* (heuristic searches with random stepwise addition and TBR branch swapping options) with all indels considered as missing data. Neighbor joining (NJ) analyses were also performed with PAUP* using the model suggested by MODELTEST. The robustness of the trees was assessed by bootstrap resampling with 1000 random NJ, MP and ML replicates.

To get a better insight into the shallow phylogeographic structures and the recent history within the clades showing a high genetic structure, we constructed a cyt *b* 95% parsimony network (Templeton et al., 1992) using TCS 1.13 software (Clement et al., 2000).

2.4. Divergence time estimations

The only calibration point available for Viperidae is based on the split induced by the uplift of the Panama isthmus, which separated populations of *Porthidium* 3.5 Mya ago (Wüster et al., 2002). Because sequences of the CR of *Porthidium* are not available, a two-gene (cyt *b* + 16S) dataset totalling 1089 bp was used to date the most recent common ancestors (MRCAs) for each clades and subclades under a relaxed molecular clock assumption using Bayesian inference (Drummond et al., 2006), as implemented in the program BEAST v1.2 (Drummond et al., 2002; Drummond and Rambaut, 2003). For this purpose sequences of *Sphenodon punctatus*, *Pogona vitticepes, Acrochrodus granulatus, Ovophis okinavensis, Porthidium ophryomegas, P. lansbergii, P. dunni and P. nasutum* (GenBank Accession Nos.: NC004815, NC006922, NC007400, NC007397, AY223580 + AF057252, AY223582 + AY223668, AY223581 + AY223667 and AY223579 + AF057251, respectively) were added to the dataset in order to use the divergence time between snakes and lizards (130–150 Mya; Carroll, 1988) as a high calibration point. Finally, the last calibration point was based on the oldest fossil record attributed to the genus *Vipera* (23.8 Mya; Szyndlar and Böhme, 1993).

These time estimates were conducted under the GTR+I+G model (the model selected by MODELTEST), a relaxed molecular clock assumption (Drummond et al., 2006), allowing the rate of substitution to vary throughout the tree in an autocorrelated manner, and with a constant population size assumption (population model with an exponential growth was also tested and produced similar results). Following a burn-in of 200,000 cycles, divergence times were sampled once every 100 cycles from 2,000,000 Markov Chain Monte Carlo (MCMC) iterations. Convergence of the chains to the stationary distribution was checked by visual inspection using TRACER (Rambaut and Drummond, 2003). In order to avoid possible local

optima, the simulation was redone three times, generating analogous results.

3. Results

The analysis of 927 bp of the cyt *b* from 59 samples revealed 44 unique haplotypes. There were 181 (19.5%) variable sites (26.6% including outgroup taxa), of which 154 (16.6%, 16.7% including outgroup) were phylogenetically informative under MP criteria. For the 16S, 400 bp were analysed, revealing 21 unique haplotypes with 27 (6.8%) variable sites (8.8% including outgroup taxa), of which 21 (5.3%, 5.5% including outgroup) were phylogenetically informative. Analysis of the CR (981 bp) revealed 47 unique haplotypes with 105 (10.7%) variable sites (14.1% including outgroup taxa), of which 89 (9.1%, 9.1% including outgroup) were phylogenetically informative. The combined data set shows 55 different haplotypes with 313 (13.6%) variable sites (17.8% including outgroup taxa), of which 264 (11.4%, 11.5% including outgroup) were phylogenetically informative under MP criteria. Uncorrected (*p*) distance divergence ranged between 0% and 5.2% within *V. ammodytes* whereas mean divergence between *V. ammodytes* and *V. aspis* reached 9.4%. Within *V. ammodytes*, signs of saturation were present only for transitions at the third codon position within the cyt *b* among ingroup taxa, and became evident between *V. ammodytes* and outgroup for CR and cyt *b* (data not shown). In addition, g_1 statistics were measured for CR ($g_1 = -0.390$: $p < 0.01$), 16S ($g_1 = -0.405$: $p < 0.01$) and cyt *b* ($g_1 = -0.463$: $p < 0.01$) as well as for each codon position of cyt *b* ($g_1 = -0.383$: $p < 0.01$ for the first position; $g_1 = -0.581$: $p < 0.01$ for the second position and $g_1 = -0.479$: $p < 0.01$ for the third position), showing that all partitions and codon positions contained significant phylogenetic signals. Therefore, saturation was not considered to be a significant factor, all nucleotide positions were used in subsequent analyses and no weighting scheme was applied to different codon positions or regions. Moreover, our results showed that the cyt *b* substitution rate was 1.64 times higher than for CR and 3.43 times higher than for 16S.

3.1. Phylogenetic analysis

The partition homogeneity test indicated a significant heterogeneity between the three mtDNA regions ($p = 0.014$). The significant value of this test is due to the relative position of the south-western, the north-western, the north-eastern and the Montenegrin clades (see below), whereas all samples belong to the same clade in the separated analyses of the three regions. Nevertheless, no significant incongruence between any pair of regions was detected (16S/cyt *b*, $p = 0.107$; 16S/CR, $p = 0.501$; cyt *b*/CR, $p = 0.084$). Therefore, analyses were conducted with a combined dataset grouping together the three regions, considering that our investigations cannot resolve the relative position between the above-mentioned clades.

For the combined dataset, the best-fit model of substitution evaluated using MODELTEST was TIM+I+G (freq. $A = 0.2944$; freq. $C = 0.2814$; freq. $G = 0.1188$; freq. $T = 0.3054$; $R(a) = R(f) = 1.0$; $R(b) = 27.49$; $R(c) = R(d) = 3.358$; $R(e) = 15.54$; proportion of invariable sites = 0.6366; gamma distribution shape parameter = 0.6486). The heuristic parsimony analysis produced 1000 equally parsimonious trees of 719 steps (CI = 0.557, RI = 0.906). Bootstrap supports of the MP, NJ and ML analyses are shown on the Fig. 2.

The trees obtained clearly show seven distinct clades of *V. ammodytes* (all with 100% bootstrap support) designated as: Montenegrin clade (samples from Montenegro), north-eastern clade (western, central and eastern Serbia, western Bulgaria), north-western clade (Italy, Austria, Slovenia, Croatia and Bosnia), south-western clade (Albania, north-western Greece), Cyclades clade (Cyclades Islands), Peloponnese clade (Peloponnese peninsula) and south-eastern clade (northern and central Greece, most of Macedonia, central and eastern Bulgaria, the southernmost part of Serbia, Turkey and northern Armenia). The Montenegrin and the north-eastern clades cluster together, even if bootstrap support was limited (between 66% and 81%). The relative position of the Montenegrin + north-eastern, the north-western, the south-western clades and the cluster formed by the Cyclades + Peloponnese + south-eastern clades could not be assessed with our data set. Indeed, the three analysed mtDNA regions gave slightly different results and the combined dataset did not support any particular relationship among them. Consequently, a basal clade could not be determined with our analyses, suggesting contemporaneous splits between all these clades. The mean genetic distance (*p*-distance) between clades varied between 2.3% and 5.2% for the combined dataset (between 3.2% and 7.6% for the cyt *b*).

Four subclades were observed within the south-eastern clade: Turkish subclade (western Turkey), Asia Minor subclade (central and eastern Turkey, northern Armenia), southern subclade (northern and central Greece, Macedonia and the southernmost Serbia) and eastern subclade (central and eastern Bulgaria). Mean distances between subclades were large (*p*-distance: 2.4%; for the cyt *b* alone: 3.8%).

The network analysis was conducted only within the north-western clade due to the high number of samples available within this clade ($n = 19$) and its high genetic structure (see Fig. 2); the other clades or subclades presented a very limited genetic structure (e.g. Montenegrin or north-eastern clade) or a reduced sample size per clade (e.g. Cyclades or Peloponnese clades). The 95% parsimony network of the northwesternmost samples suggested that they derived from Middle Dalmatia (Fig. 3). Two different post-glacial colonisation routes were detected, one clustering the northwest Croatian and most of the Slovenian samples, the other one regrouping all north-Italian and Austrian samples.

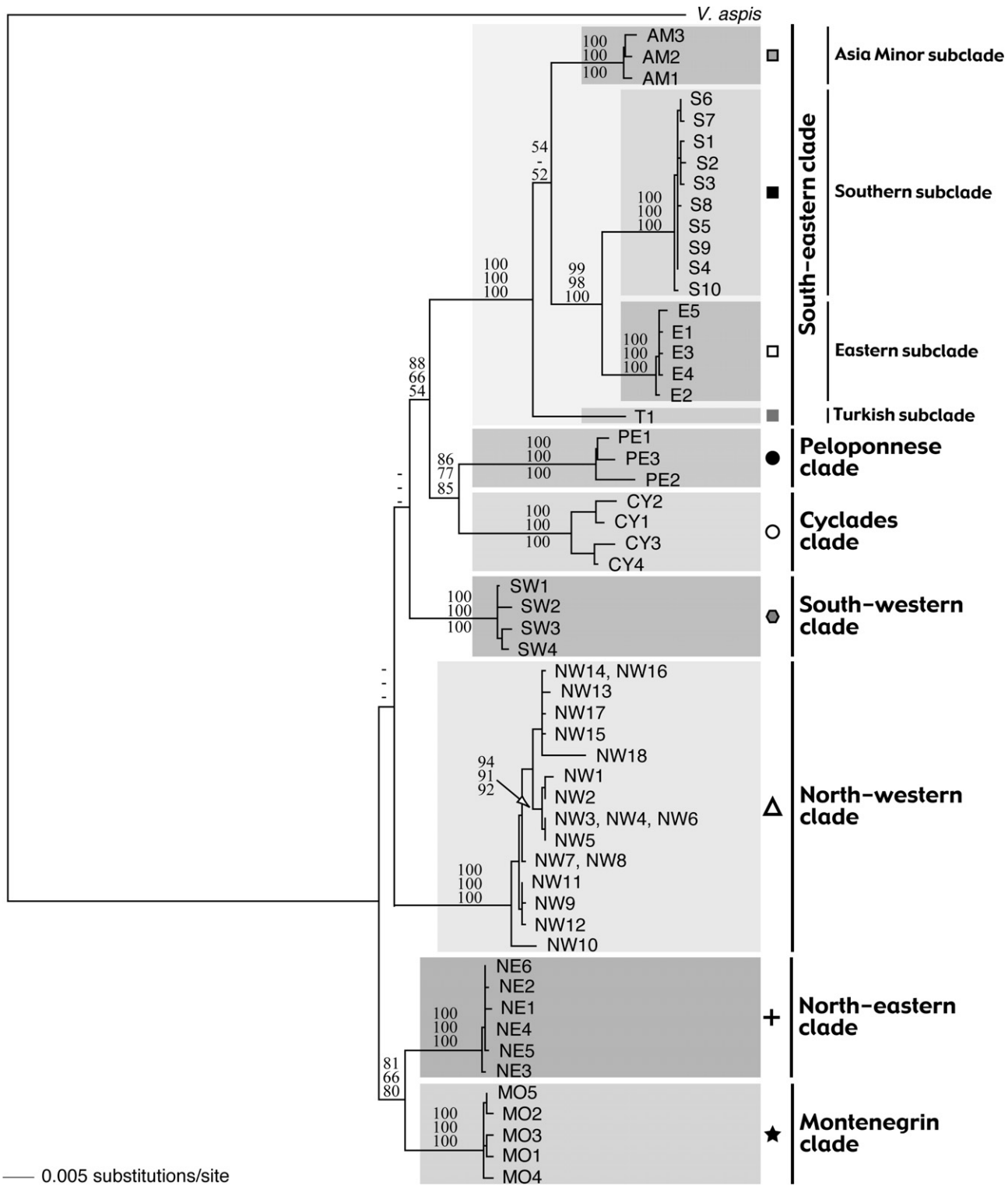

Fig. 2. Maximum likelihood tree for combined dataset (16S, cytochrome *b* and the control region of the mtDNA, regrouping 2308 bp) of *Vipera ammodytes*. Values of bootstrap support are shown for nodes found in more than 50% of 1000 trees of Neighbor Joining, maximum parsimony and maximum likelihood analyses, respectively. Individual ID and symbols correspond to sample localities in Fig. 1.

3.2. Divergence time estimations

According to the evaluation obtained with BEAST, the first splits within *V. ammodytes* occurred during the Early Pliocene (4.1 Mya, 95% highest posterior density, HPD: 3.4–4.9 Mya), separating the Montenegrin + north-eastern, the north-western, south-western and the Peloponnese + Cyclades + south-eastern clades. The divergence between the Peloponnese and the Cyclades clades was evaluated to take place in the Mid Pliocene (3.5 Mya, 95%

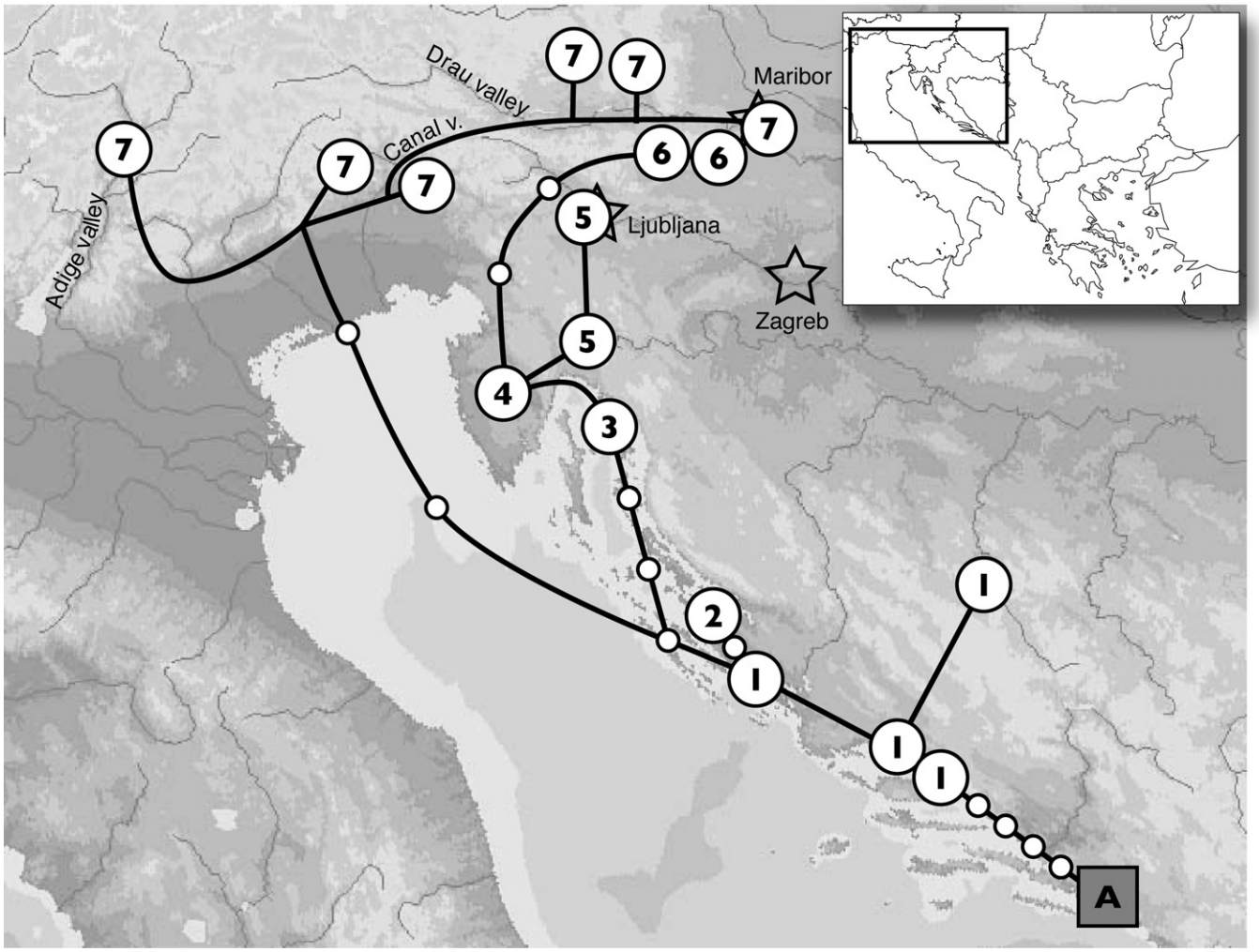

Fig. 3. Parsimony network of cytochrome *b* haplotypes estimated under the 95% statistical limits of parsimony using the algorithm of (Templeton et al., 1992) indicating the post-glacial colonisation of the north-westernmost part of the area. Numbered circles: recorded haplotypes (for localities see Appendix A). Small circles stand for missing haplotypes. (A) The haplotype assumed as ancient for post-glacial spread to the north.

HPD: 2.6–4.4 Mya), whereas the age of the MRCA within the south-eastern clade was estimated to 2.7 Mya (95% HPD: 2.0–3.5 Mya; see Fig. 4). All splits within each clade and subclade took place in the Pleistocene, particularly during the last 0.7 Mya.

4. Discussion

4.1. Phylogeographic reconstruction

Overall, we found considerable genetic diversity in *V. ammodytes* within the Balkan peninsula. According to the fossil data, the ancestor of the "*Vipera aspis* complex" appeared in central Europe at the beginning of the Miocene and continued to persist there until the end of the Pliocene (Szyndlar and Rage, 1999). The Balkan peninsula in the Miocene was characterised by changes in its relief, the loss of ancient corridors and the establishment of new ones, as well as by climatic and vegetation changes (Andjelković, 1988; Oosterbroek and Arntzen, 1992; Rage et al., 2003). The ancestor of *V. ammodytes* probably colonised the Balkans during this period. Our results suggest an early Pliocene splitting of *V. ammodytes* into four groups, but our analyses were not able to determine where this event occurred, since basal clades were different according to the mtDNA regions we analysed (Fig. 2).

The isolation between the Peloponnese, the central Cyclades Islands and the Greek mainland during most of the Pliocene (3–4 Mya; Dermitzakis, 1990) corresponds with the divergence between the Cyclades and the Peloponnese clade estimated by this study (3.5 Mya, 95% HPD: 2.6–4.4). During the late Pliocene, individuals from the south-eastern clade colonised Turkey up to the Caucasus Mountains and split into several subclades possibly due to isolation by distance.

Interestingly, the presence of well supported mitochondrial clades in Serbia, Montenegro and Albania/north-western Greece (*V. ammodytes* from these regions were usually treated as a part of the nominate subspecies), as well as from the Peloponnese peninsula (always considered as

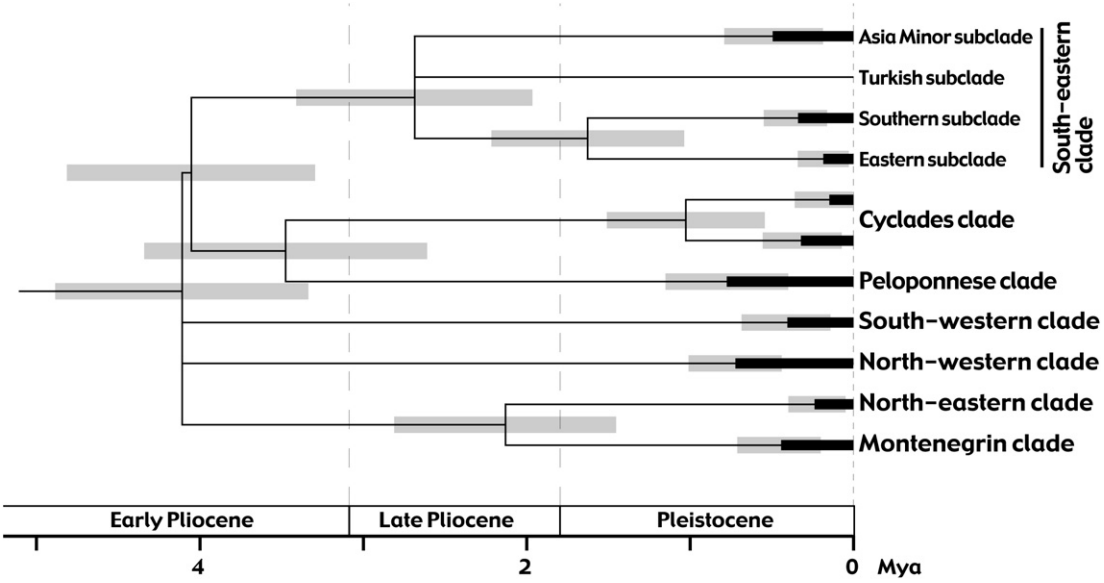

Fig. 4. Dating of the most recent common ancestors (with % highest posterior density in grey) computed using BEAST (Drummond et al., 2002; Drummond and Rambaut, 2003) for the deepest splits. The black bars correspond to the timing of the divergence within clades estimated using BEAST.

belonging to the subspecies *meridionalis*) has never been suggested from morphological data. The most recent studies based on morphological data showed no differentiation between the above-mentioned populations (Tomović, 2006; Tomović and Džukić, 2003). However, recent studies, for several animal and plant taxa suggest high levels of genetic diversity in the Balkan peninsula, with the occurrence of several distinct refugia during the last glaciations or before. For instance, Magri et al. (2006) observed genetically different populations of beech *Fagus sylvatica* and proposed the occurrence of three distinct glacial refugia in the Balkans peninsula. Sotiropoulos et al. (2007) found high mitochondrial diversity in the newt *Mesotriton alpestris* within the Balkan region (at least 6 distinct refugia). Another newt, *Triturus vulgaris,* also shows a high genetic diversity in the Balkan peninsula (Babik et al., 2005). Finally, Wallis and Arntzen (1989) estimated that the split within the *Triturus cristatus* group occurred during the Pliocene, as observed for *V. ammodytes*. Additionally, the high genetic diversity on the Adriatic coast detected in *V. ammodytes* was also observed for the Martino's vole (*Dinaromys bogdanovi*) by Krystufek et al. (2007) or for the Dalmatian wall lizard (*Podarcis melisellensis*) by Podnar et al. (2004). Thus, as for the Iberian and, to a lesser extent, the Italian peninsulas, the Balkan region seems to possess high levels of endemism. The large habitat heterogeneity and the numerous mountain formations have led to a genetic differentiation among taxa, resulting in a hot spot of endemism due to the isolated "refugia-within-refugia" (see Sotiropoulos et al., 2007).

4.2. Impact of the Quaternary glaciations

Our results suggest that the main differentiations between clades occurred during the Pliocene, whereas the glaciations of the Quaternary only influenced the genetic diversity within clades. Indeed, most intra-clade and intra-subclade genetic distances are low (up to 0.5% for the cyt *b*) except for the north-western clade (up to 1.6%), the Peloponnese clade (up to 1.3%) and the Cyclades clade (up to 1.7%). Moreover, the MRCAs for all the remaining clades and subclades were estimated to originate from less than 500,000 years. This high homogeneity implies that most of the clades underwent bottlenecks during the last glaciations, probably due to limited refugial areas. It is possible that competition with vipers of the "*V. berus* group", which are more adapted to low temperature environments and expanded their distribution during the Pleistocene, strongly influenced the fragmentation of the "*V. aspis* group" (Szyndlar and Rage, 1999). Consequently, the glaciations of the Quaternary have probably fragmented *V. ammodytes* populations into several refugia in the Balkans. The last glaciations had a particularly marked impact on the internal structure of the north-western clade, where a significant genetic substructure was detected (see below).

On the opposite, *V. ammodytes* from the Cyclades clade were isolated on different islands during periods of high sea level and, consequently, evolved separately, leading to the high differentiation observed between the northern and southern Cyclades Islands (samples CY1 + CY2 and CY3 + CY4, see Fig. 2).

4.3. Substructure in the north-western clade

As a result of the harsh conditions during the last glacial maximum, northern Dalmatia was probably not inhabited by *V. ammodytes*. Therefore, all populations currently inhabiting this area are the result of a post-glacial colonisation from southern refugial areas. Although relatively high

genetic differences within this clade seem to indicate old differentiation (0.73 Mya according to the estimation based on BEAST), the TCS results suggest that these differences are due to post-glacial expansions. This inconsistency could be explained by a higher mutation rate across the genealogical timescale when the time estimate is lower than 1 Mya (Ho and Larson, 2006). In fact, Ho and Larson (2006) demonstrated a high level of discrepancy of the timing between date estimates based on genetic analyses with archaeological estimates especially when archaeological events occurred less than 15,000 years ago, which can be the case in our study. During the last glaciation, the northern limit of the distribution was obviously more southernly than the present distribution and probably near Dalmatia. Therefore, post-glacial colonisation proceeded from southern to northern. The TCS analysis indicated two different colonisation routes out of middle Dalmatia up to the north. One of the routes could have run along today's north Croatian offshore islands, then across Istria and ending in northern Slovenia. According to this hypothesis, the other route reached north-eastern Italy, possibly crossing the north Adriatic basin, presently flooded. The Adige valley (Italy) could be colonised upstream and the Drau valley (Austria) could be reached over the Canal valley, from where the colonisation progressed downstream, meeting the outpost of the other lineage in the Maribor area (northern Slovenia, Fig. 3). Based on the ML tree, the high homogeneity within the second route confirms this pattern. However, more samples should be analysed in this region in order to be able to test complementary hypotheses such as genetic diversity reduction from the refugial area to the peripheral (or newly colonised) locations.

4.4. Taxonomical implications

Vipera ammodytes holds several subspecies with dubious validity (see Tomović and Džukić, 2003; and references therein). The results of our study clearly showed that populations from the north-western clade (Italy, Austria, Slovenia, Croatia and Bosnia) are genetically close, even if small intra-clade structuring probably resulted from the accumulation of substitutions during the post-glacial expansion. In particular, the mtDNA sequences from all samples of the subspecies *V. a. ruffoi* in the Adige valley (Italy) and *V. a. gregorwallneri* in the southernmost Austria (Bruno, 1968; Sochurek, 1974), which were described as colour morphs, as well as the samples of the subspecies *ammodytes* from north-eastern Italy (Friuli), were identical. Whereas the aims of this study were not to propose modifications to the present systematic of *V. ammodytes,* both genetic and morphological studies (this study and Tomović, 2006) confirm that these subspecies have to be considered as synonyms of *V. a. ammodytes* (as also Heckes et al., 2005).

The easternmost portion of the species range (the Asian part of Turkey, Armenia and Georgia) is inhabited by *V. a. transcaucasiana* (e.g. Başoğlu and Baran, 1980; Bergman and Norström, 1994; Derjugin, 1901; Eiselt and Baran, 1970; Kutrup, 1999; Nilson et al., 1988). This subspecies was sometimes considered as a separate species (Nilson et al., 1999; Obst, 1983). Relatively high immunological distances (Herrmann et al., 1987) between *V. a. meridionalis* and *V. a. transcaucasiana* seemed to support this opinion. Heckes et al. (2005) abandoned this opinion considering *transcaucasiana* as a subspecies. Our genetic analyses, as well as Tomović (2006) confirm its status as subspecies since the studied samples of this taxon were grouped within the south-eastern clade. A distinct history and several morphological adaptations observed within this subspecies (compared with the other members of the south-eastern clade) suggested that the *V. ammodytes* specimens located on the far east of its distribution range should be regarded as a separate ESU (see Moritz, 1994). However, due to a lower sample size of this subspecies compared to the other *ammodytes* taxa (both in this and in previous morphological studies; Tomović, 2006), we have to remain cautious over its taxonomic status.

The mitochondrial DNA tree is not in agreement with the traditional taxonomic subdivisions based on morphological characters of *V. ammodytes* in southern Balkans. Morphological features do not seem to suitably trace the history of the population groups for this species. Indeed, the conclusion of Tomović (2006) suggested a clinal morphological variation within *V. ammodytes*, possibly related to environmental variations. Relationships between temperature or altitude and morphology have already been demonstrated in some snakes species (e.g. bamboo vipers species [*Trimeresurus*] in Taiwan, Castellano et al., 1994; Sanders et al., 2004, 2006; or long-nosed snakes [*Rhinocheilus lecontei*], Manier, 2004). Thus, the previous subdivisions based only on morphological characters do not reflect historical differentiations within *V. ammodytes* populations. However, nuclear markers should be studied to confirm the observed pattern of genetic structure and to verify that the morphological homogeneity is not due to a genetic homogenisation due to male-only dispersal. In addition, morphological analyses should be conducted using the genetic splits found here in order to identify morphological characters (if some occur) associated with the evolutionary history of this species and not related to other external factors.

To conclude, the nose-horned viper is similar to other reptile and amphibian species (e.g. Poulakakis et al., 2005; Wallis and Arntzen, 1989) in displaying a huge genetic variability in the Balkan peninsula and the Hellenic region. Even if there is some agreement between the patterns of morphological and genetic structuring, the taxonomy of this species should be completely revised by combining molecular analyses of nuclear genes, intra-population genetic diversity investigations and additional morphological examinations. Our study established that the main cladogenetic events for this species occurred before the Quaternary glacial period. Thus, Pleistocene climatic changes have influenced the genetic diversity only within

most of the main clades. Finally, the large genetic diversity within *V. ammodytes* demonstrates the considerable complexity and high number of potential refugia in the Balkan peninsula, calling for additional phylogeographic investigations in species with similar distributions.

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Appendix A

Samples of *Vipera ammodytes* used in the present study

Subspecies	Locality	Country	Code	*
V. a. ammodytes	Friuli, Musi	Italy	NW2	7
	Friuli, Villa Santina	Italy	§	7
	Maribor	Slovenia	NW6	7
	Velenje	Slovenia	NW7	6
	Huda luknja	Slovenia	NW8	6
	Ljubljana, Javor	Slovenia	NW9	5
	Snežnik	Slovenia	NW10	5
	Rovinj	Croatia	NW11	4
	Krk Island	Croatia	NW12	3
	Bokanjac, Zadar	Croatia	NW13	2
	Zadar	Croatia	NW14	1
	Vaganac River Canyon	Bosnia	NW15	1
	Mosor Mt.	Croatia	NW16	1
	Omiš	Croatia	NW17	1
	Slano	Croatia	§	A
	Near Dubrovnik	Croatia	NW18	
	Lisina spring, Beljanica Mt.	Serbia	NE1	
	Village Kaluger, Belogradčiško	Bulgaria	NE2	
	Village Brod, Crna Trava	Serbia	NE3	
	Šumanska River, Medveđa	Serbia	NE4	
	Village Brzeće, Kopaonik Mt.	Serbia	NE5	
	Uvac River Canyon, Zlatar Mt.	Serbia	NE6	
	Village Tepca, Tara River Canyon	Montenegro	MO1	
	Mrtvica River Canyon	Montenegro	MO2	
	Cetinje	Montenegro	MO3	
	Budva	Montenegro	MO4	
	Skadarsko Lake	Montenegro	MO5	
V. a. gregorwallneri	Carinthia, Friesach	Austria	NW3	7
	Carinthia, Friesach	Austria	NW4	7
	Carinthia, Frantschach	Austria	NW5	7
V. a. meridionalis	Vlore	Albania	SW1	
	Corfou Island	Greece	SW2	
	Igoumenitsa	Greece	SW3	
	Near Ioannina	Greece	SW4	
	Crnovska River, Trgovište	Serbia	S1	
	Nov Dojran	Macedonia	S2	
	Prespa Lake	Greece	S3	

(*continued on next page*)

Appendix A (*continued*)

Subspecies	Locality	Country	Code	*
	Chalkidiki	Greece	S4	
	Olymp	Greece	S5	
	Mount Ossa	Greece	S6	
	Olymp, Damasi	Greece	S7	
	Lamia	Greece	S8	
	North Evvia	Greece	S9	
	Nafpaktos	Greece	S10	
	Basin of Feneos	Greece	PE1	
	Kiparissia	Greece	PE2	
	Lakonia, Kardamili	Greece	PE3	
	Tinos Island	Greece	CY1	
	Siros Island	Greece	CY2	
	Naxos Island	Greece	CY3	
	Ios Island	Greece	CY4	
	Sapanca	Turkey	T1	
	Village Zara east of Sivas	Turkey	AM1	
V. a. montandoni	Village Sadovec, Plevensko	Bulgaria	E1	
	Village Nadežden, Harmanlijsko	Bulgaria	E2	
	North costal area	Bulgaria	E3	
	Carevo	Bulgaria	E4	
	Ahtopol	Bulgaria	E5	
V. a. ruffoi	Bozen	Italy	NW1	7
V. a. transcaucasiana	Aralik	Turkey	AM2	
	Kura valley	Armenia	AM3	

The subspecies were defined according to relevant literature. *Numbers correspond to those in Fig. 3. §Only the cytochrome *b* gene was sequenced for this sample.

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