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#### ORIGINAL ARTICLE

# Genome-wide single nucleotide polymorphisms reveal recurrent waves of speciation in niche-pockets, in Europe's most venomous snake

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#### Abstract

Within the Balkan Peninsula, topographic and climatic agents have promoted biodiversity and shaped the speciation history of many ectotherms. Here, we targeted an iconic European reptile, the nose-horned viper species-complex (Vipera ammodytes), and explored its spatial and temporal evolution. We (i) utilized genome-wide single nucleotide polymorphisms to infer genetic structure and build a time-calibrated speciestree, and (ii) applied species distribution modelling with niche-divergence tests among major phylogenomic clades. Geographically structured genetic diversity was found. Cycles of recurrent isolation and expansion during glacial-interglacial periods led to allopatric speciation and to secondary contacts and formation of multiple hybrid zones throughout the Balkan Peninsula. Deep divergence is still detected among populations separated by old and imminent biogeographical barriers (Pindos Mountain Range, the Cyclades islands, etc.), but in most cases speciation is incomplete. At the other end of the speciation continuum, we recognize two well-differentiated lineages, currently lacking any evidence of gene flow; one is distributed in the Northwestern Balkans and the other in the Southeastern Balkans, further expanding into Asia. Despite their split 5 million years ago, there is no evidence of ecological divergence, as speciation probably occurred in niche-pockets of analogous environments. These two lineages probably represent different species, while V. transcaucasiana does not merit species status. By comparing the genomic phylogenies to an updated mitochondrial one, we propose an evolutionary scenario that resolves all mitonuclear conflicts, according to which the history of the V.ammodytes species-complex was shaped by complex processes, including a major event of introgressive hybridization with asymmetric mitochondrial capture.

#### KEYWORDS

Balkan subrefugia, ddRAD, genomics, niche modelling, SNPs, Vipera ammodytes species-complex

Evanthia Thanou and Panagiotis Kornilios contributed equally to this study.

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#### 1 | INTRODUCTION

The Balkan Peninsula is considered a biodiversity hotspot, in terms of species and phylogeographical diversity (Griffiths et al., 2004). The primary factors that have generated, shaped and maintained the high levels of biodiversity are its old and intense geoclimatic history and rich topographic heterogeneity, including the existence of many islands and ragged mountains. Due to these characteristics, this region has served as an important radiation centre and a refugium for the survival of species and evolutionary lineages (Hewitt, 2000). Additionally, the topographic complexity has allowed subregions to act as climatic pockets or "refugia within refugia" during the Quaternary climatic fluctuations (Nieto-Feliner, 2011). More specifically, the topographic relief, especially of the southern and western parts of the peninsula, is extensively shaped by two mountain systems: the Dinarides in the north and the Hellenides, mainly the Pindos Mountain Range (hereafter Pindos), in the south (Figure 1 and Figure S1). These systems act as strong biogeographical barriers

and define areas of high species richness and endemism for reptiles and amphibians (Jablonski et al., 2021; Sillero et al., 2014).

The spatial genetic structure of a species often reflects the intensity and duration of exposure to climatic changes and the effect of biogeographical barriers (Lawrence & Fraser, 2020), as especially observed in ectotherm reptiles and amphibians (e.g., Dufresnes et al., 2018; Gvoždík et al., 2013; Mezzasalma et al., 2018; Wielstra et al., 2013). Climatic fluctuations, such as those of the Plio-Pleistocene, may lead to alternating periods of isolations and secondary contacts (Hewitt, 2000). The allopatric isolations, mostly due to local extinctions, promote different levels of speciation, while secondary contacts may form hybrid zones with gene flow between lineages expanding from refugia (Pflugbeil et al., 2021). Although speciation had been historically thought to occur without hybridization, the latter is now considered a common element of the speciation process (Taylor & Larson, 2019). Consequently, most recent studies that use molecular genetic markers, such as genomic ones, return evidence of introgression between highly diverged



FIGURE 1 (a) Map of the approximate distribution of the Vipera ammodytes species-complex. The two currently recognized species, the European V. ammodytes and the Asiatic V. trancaucasiana, are shown with red and green, respectively. Areas of unconfirmed distribution are noted with question marks (?) and sites of fossil records with red stars (see Šmíd & Tolley, 2019). (b, c) Maps showing the collection sites for the samples (dots) or mitochondrial sequences (squares) used in the molecular analyses. Cytochrome b (*cytb*) sequences originally published in Ursenbacher et al. (2008) are given with their respective GenBank Accession Numbers. Samples used for the preparation of genomic libraries and additional cytb sequences produced in this study are marked with numerical codes, as in Table S1 (see there for country codes and sample details). Geographical areas (Evvoia, Cyclades Islands and Peloponnesos) and features (Pindos Mountain Range; dashed red line) discussed in the text are shown. [Colour figure can be viewed at wileyonlinelibrary.com]

populations, which, in turn, may generate mitonuclear discordance observed as conflict between analyses of nuclear and mitochondrial data (Andersen et al., 2021).

Next-generation sequencing (NGS) and the methodological advances in producing reduced-representation libraries, especially in the absence of known reference genomes, have allowed the study of genome-wide patterns of differentiation along the speciation continuum (Feder et al., 2012). The analysis of genome-wide polymorphisms helps investigate complex diversification histories that may include gene flow, which traditional single-copy nuclear and mitochondrial markers cannot. When such approaches are applied, their results often deviate from the diversity patterns outlined by established taxonomies. In many cases, biodiversity has been overestimated and taxonomic inflation is recorded (e.g., Chan et al., 2022), while in others a significant underestimation of the existing biodiversity is found in the form of cryptic species or lineages, hiding more complex evolutionary histories (e.g., Psonis et al., 2018).

The vipers of the genus Vipera Laurenti, 1768 constitute a monophyletic group within the subfamily Viperinae (Serpentes, Viperidae) and present an example of a complex diversification history with contradictory diversity patterns. This genus of Asian origin (Šmíd & Tolley, 2019) includes 21 currently recognized species, the outcome of a long and complex taxonomic process (Freitas et al., 2020). The earliest molecular phylogenies based on mitochondrial markers (mtDNA) detected three major lineages, the Pelias complex, the V. aspis complex and the V. ammodytes complex (Garrigues et al., 2005). Freitas et al. (2020) further showed that the group exhibits both taxonomic inflation and cryptic diversity depending on the lineage. The Pelias group, distributed throughout most of Europe and Asia, includes at least 14 species, which is probably an overestimation. The V. aspis group includes three endemic species from the Iberian Peninsula, the Italian Peninsula and northwest Africa, while cryptic subspecies were recently described (Martínez-Freiría et al., 2021). Finally, the V.ammodytes complex has a wide geographical distribution in southeast Europe and a small region in west Asia: it can be found from as west as Italy and southern Austria to as east as the Caucasus Mountains and the Transcaucasian region along the northern Anatolian coast. A major part of its range is in the Balkan Peninsula, including the Aegean islands (Figure 1a).

The nose-horned viper, V.ammodytes, is one of Europe's most dangerous venomous snakes, officially listed as a Category 1 medically important snake for Europe (Di Nicola et al., 2021). Accordingly, the bulk of published literature regarding the nose-horned viper relate to venom studies, with other aspects of its biology and lifehistory relatively neglected. The group has undergone numerous taxonomic rearrangements for more than 250 years and presents a complex situation with regard to the included species, subspecies, morphotypes and presumed cryptic species (see Section 4). Mitochondrial phylogenies are in conflict with aspects of its current taxonomy; they demonstrate a deep phylogenetic structure similar or greater to that of the other two Vipera subgroups and suggest the existence of several cryptic species (Freitas et al., 2020; Ursenbacher et al., 2008). However, this hypothesis has not been further tested with the analysis of other genetic markers besides mtDNA, and especially genomic ones.

The current taxonomy of European vipers has been traditionally based on morphological traits which show high variability even at the intraspecific level and could be related to adaptive processes (Freitas et al., 2020 and references therein). Within the *V. ammodytes* species-complex, there is evidence of a north-to-south cline of morphological differentiation along the Balkans (Tomović et al., 2010), while some insular populations exhibit dwarfism (e.g., Cyclades). Because adaptation to local climatic conditions is expected to play a significant role in the diversification of ectotherms, niche reconstructions against a known phylogeography may provide useful insight into their evolutionary history (see Wielstra et al., 2013). In *V. ammodytes*, phylogeographical inferences have not been so far complemented with ecological data to evaluate whether genetic splits coincide with niche segregation.

The nose-horned viper offers an ideal model to test how temporal and spatial agents can promote and shape the speciation history of ectotherms in the Balkans and the Southern Mediterranean. Here, we implement an integrative approach to investigate the evolutionary history of the V. ammodytes species-complex by bringing together genomic and ecological data, and an updated maternal phylogeny. Based on known diversification patterns from species distributed in the Balkan Peninsula, including results from mtDNA analyses for the nose-horned viper (Ursenbacher et al., 2008), we predict that the Plio-Pleistocene history of this region was the principal driver of its speciation history. To test our hypothesis, we utilize NGS and the double-digest restriction site-associated DNA methodology (ddRAD) to sequence a large number of genome-wide loci, in order to (i) reconstruct a time-calibrated phylogenomic species-tree, (ii) infer the spatial genetic structure and (iii) identify barriers and corridors of gene flow and introgression. We combine the observations based on both biparental and maternal genetic data with species' distribution models (SDMs) and niche-overlap analyses to further test the hypothesis of ecological divergence among major lineages and infer biogeographical scenarios for the evolution of our model organism.

#### 2 | MATERIALS AND METHODS

#### 2.1 | Genetic sampling

Currently recognized species and subspecies of the target group, all involved biogeographical regions and all known mitochondrial lineages were analysed with a total of 149 DNA samples (Table S1; sampling localities shown in Figure 1); 57 ingroup samples and two *Vipera berus* were used as the outgroup in the genomic data sets, and 142 ingroup sequences with five *V. berus* and two *V. aspis* were used in the mtDNA data set.

# 2.2 | ddRAD library preparation, sequencing and bioinformatics

We collected ddRADseq data following the protocol, barcodeadaptors and indices of Peterson et al. (2012), with the wet-laboratory procedure for library preparation and sequencing detailed in Thanou et al. (2020). Raw Illumina reads were processed with IPYRAD version 0.7.30 (Eaton, 2014); details on filtering parameters and resulting data sets are given in Table S2. The IPYRAD pipeline ran each time a new data set was generated, for example including or excluding outgroups. We used two levels of missing data, expressed as the minimum number of individuals with data for a given locus: 100% (each locus present in all samples) and 75% (each locus present in at least 75% of the samples). Higher levels of missing data will increase the number of loci and single nucleotide polymorphisms (SNPs) in the final data matrix but can also negatively influence some of the downstream analyses. Two types of final data matrices were generated for different analyses that included either the entire ddRAD locus ("ddRAD" data sets) or by choosing one random SNP from each putatively unlinked locus ("uSNP" data sets).

#### 2.3 | Genomic population-clustering and admixture

For the genetic clustering we implemented STRUCTURE version 2.3.4 (Pritchard et al., 2000), and the uSNPs-100 genomic data set of all ingroup individuals. We ran preliminary analyses testing different models: independent/correlated allele frequencies, admixture/ nonadmixture, degree of admixture between clusters (prior *a*), and distribution of allele frequencies (prior  $\lambda$ ). For the final analysis, we performed 10 runs of  $5 \times 10^5$  iterations each and a 25% burn-in, the independent/no admixture model ( $a=0.01\pm0.025$ ,  $\lambda=0.33$ ) and tested up to 12 clusters (*K*). Following a hierarchical approach, we repeated STRUCTURE analyses on the major genetic clusters found within our study system, applying the correlated/admixture model ( $a=0.01\pm0.05$ ,  $\lambda=0.36$ ) and testing for K=1-10. To visualize clusters and determine Evanno's optimal *K* ( $K_{opt}$ ; Evanno et al., 2005), we used the CLUMPAK online web server (Kopelman et al., 2015).

# 2.4 | Phylogenomic trees and estimation of divergence times

We estimated a species tree under the Bayesian multispecies coalescent framework of SNAPP version 1.5.1 (Bryant et al., 2012) implemented in BEAST2 version 2.6.3 (Bouckaert et al., 2014) and followed Stange et al. (2018) to transform the SNAPP species-tree analysis into a strict molecular-clock analysis. Both simulation and empirical data (Stange et al., 2018) have shown that SNAPP molecular dating returns very accurate inferences even when each population is represented by only one individual. In this context, and in order to minimize computation time and maximize the number of SNPs in our data set, we represented each population with two individuals, except for two — MOLECULAR ECOLOGY – WIL

populations that only include one individual each. To avoid model violations, since SNAPP assumes no gene flow, we included nonadmixed individuals (membership probability Q>99% according to structure). Nevertheless, in our final analysis, we also included one representative from the Caucasus, despite its detected admixture (see Section 3) in order to obtain information on its phylogenetic position. The analysis ran with 11 populations/species, including *V. berus*, 20 samples in total and the genetic-clustering results used for population assignments. We performed two independent runs with a chain length of 10<sup>7</sup> generations, sampling every 250 generations, using both the uSNPs-100 data set and the uSNPs-75 data set, transformed into biallelic format. We checked convergence (effective sample size (ESS>200)) and determined burn-in (10%) with TRACER version 1.6 (Rambaut & Drummond, 2009). A maximum clade credibility tree (MCC) was summarized with TRECENVENTATOR.

The root of the species tree, that is the most recent common ancestor (MRCA) of V. berus and the V. ammodytes complex, was calibrated based on published estimates. Several studies that utilized mtDNA, mtDNA+nuclear DNA, or genomic markers have resulted in similar estimations for the divergence between V. berus and V. ammodytes, for example ~12.5 million years ago (Ma) (Wüster et al., 2008), 15.2 Ma (Zheng & Wiens, 2015), 16.1 Ma (Alencar et al., 2016) or 16.6 Ma (Freitas et al., 2020). In a recent multimarker study, specifically aimed at a more accurate and reliable molecular-clock calibration for vipers (Šmíd & Tolley, 2019), the time of this divergence was estimated at 13.4 Ma (11.5-15.4), attributed to the mid-Miocene cooling event 14 Ma. In this context, we have calibrated the respective node using a normal distribution with a mean value of 14Ma and a standard deviation of 1.0 Ma, so that the resulting normal distribution lies between 11 and 17 Ma. in order to accommodate all reported ages.

We used the coalescent method of svDqUARTETS version 1.0 (Chifman & Kubatko, 2014), implemented in PAUP\* version 4.0a (Swofford, 2003) to infer phylogenomic relationships among individuals. We evaluated all possible quartets and statistical support with a nonparametric bootstrapping of 1000 replicates. We included nonadmixed individuals (44 in total) and two *V. berus* as outgroup. The analysis ran both with the uSNPs-100 and the uSNPs-75 data set.

Finally, a maximum likelihood (ML) tree was constructed using the concatenated ddRAD loci with IQ-TREE 1.5.5 (Nguyen et al., 2015). We used the "Auto" option for the best-fit substitution model and tested nodal support via 10,000 replicates of SH-aLRT tests (Guindon et al., 2010) and 10,000 ultrafast bootstrap alignments (Minh et al., 2013). Analysis ran without admixed individuals (44), *V. berus* as the outgroup and the ddRAD-75 data set.

#### 2.5 | Introgression among clades

We investigated potential gene flow among our inferred phylogenetic clades using TREEMIX version 1.13 (Pickrell & Pritchard, 2012). TREEMIX estimates a bifurcating ML population-tree and then adds

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migration events (*m*) leading to genetic admixture, improving the likelihood fit. We used the uSNPs-75 genomic data set of all individuals (nonadmixed and admixed) of the *V.ammodytes* complex. We assigned individuals to populations following the results of the population clustering but also incorporating the relationships found in the phylogenomic and species-tree analyses. The root position was selected based on the topology of the phylogenomic trees (see Section 3). We used blocks of 100 uSNPs and performed analyses with 100 bootstrap replicates. We first reconstructed an ML tree and progressively added migration events (*m*) up to a total of 10. Results were summarized with the R package OPTM (Fitak, 2021) to determine the optimal number of *m* by (i) examining the average increase in variance explained with each added migration, (ii) examining the change in the likelihood values and (iii) applying the Evanno method.

Additionally, we tested for genomic admixture, as opposed to "treeness," by using f-statistics (Patterson et al., 2012; Reich et al., 2009). Specifically, we applied the three-population (f3) and four-population (f4) tests, incorporated in TREEMIX, again using blocks of 100 uSNPs. In the f3 test (A; B, C), when the estimated f3 statistic and corresponding z score (the deviation of the f3 statistic from zero in units of the standard error) are negative, then population A does not form a simple tree with B and C, indicating genomic admixture between the two. In the f4 test (A, B; C, D), nonzero values of z ( $\geq$ 3 or  $\leq$ -3) indicate introgression.

# 2.6 | Mitochondrial gene-tree, single-locus species delimitation and alternative topologies

The mitochondrial gene cytochrome *b* (*cytb*) was PCR (polymerase chain reaction)-amplified and sequenced with primers L14910 and H16064 (Burbrink et al., 2000), following standard procedures. Our final data set included 76 new and 73 published sequences (149 in total), end-trimmed to a length of 932 bp, and aligned with CLUSTALX version 2.0.12 (Larkin et al., 2007) under default parameters.

We reconstructed ML trees with IQ-TREE with the "partitionfinder" and "Auto" options to determine the best partitioning scheme and best-fit substitution model for each partition (codon positions). Nodal support was tested via SH-aLRT tests with 10,000 replicates, 10,000 ultrafast bootstrap alignments and 1000 standard bootstrap alignments (Felsenstein, 1985). The tree was rooted with *V.berus* and *V.aspis*. We used the resulting mtDNA ML tree, after outgroup-cropping, as input for the delimitation of mtDNA genetic clusters with the multirate Poisson tree processes model (MPTP; Kapli et al., 2016). We combined two independent runs of 10<sup>6</sup> generations each, sampling every 1000 generations and with a 10% burn-in.

Based on the results from the phylogenomic tree analyses, we tested whether alternative topologies of the mtDNA tree are rejected or not. We performed independent tests for each hypothesis, instead of constraining the entire mtDNA tree to the topology of the genomic ones, in order to identify specific mitonuclear discordances. We pruned the mitochondrial data set to include the 44 nonadmixed individuals used in phylogenomic analyses, constrained specific relationships on the mtDNA tree and compared the constrained trees to an unconstrained one, using all the topology tests provided in the IQ-TREE package.

#### 2.7 | Niche modelling and niche divergence

Our final data set of georeferenced occurrences was compiled from the Global Biodiversity Information Facility database (GBIF, 2021), published records and our own unpublished data, resulting in 431 presence-points after trimming of duplicates and a thinning process to remove spatial bias (details in Figure S5 and Table S5). SDMs ran in R version 4.0.2 (R Development Core Team, 2020), with MAXENT version 3.4.1 (Phillips et al., 2017; http://biodiversityinformatics.amnh. org/open\_source/maxent). We downloaded data of the 19 available climatic variables of present environmental conditions from the WorldClim Database version 1.4 (Hiimans et al., 2005; http:// www.worldclim.org) and elevation from the SRTM Database version 4.1 (Jarvis et al., 2008; http://srtm.csi.cgiar.org), and derived data of slope and slope aspect. Layers were resized, set at a  $5 \times 5$ -km spatial resolution, and cropped to the study area, which was based on the current distribution of the V.ammodytes species-complex. Exploratory models were constructed and evaluated with 100 bootstrap replicates to define the optimal model parameterization and retain only the noncorrelated variables (Pearson's coefficient  $r \le .75$ ). Final models were built on the variables showing the greatest predictive contribution, that is nine climatic and one topographical (Table S5). For each model, we estimated the average area under the curve (AUC) as a measure of fit, using pseudo-absences chosen at random from the study area, and setting AUC>0.80 for efficient models.

Niche modelling focused on the major phylogroups revealed by our analyses (see Section 3), which may, however, extend over areas with different climatic conditions. To account for this, SDMs were first constructed on four subsets, representing records from (i) all islands constituting the Cyclades Island Group; (ii) the Asiatic range of distribution, hereafter named "Asia"; (iii) the European northwestern area of distribution, named "North," which included all points located north of the 41° parallel (north of Tirana, Albania) and west of the 24° meridian (west of Sofia, Bulgaria); and (iv) the European southeastern area of distribution, named "South," which includes all the remaining distribution south of Tirana and east of Sofia (including European Turkey). Records within areas of putative contact between North and South were excluded (see Section 3). Then, subsets that showed high niche similarity were grouped and the modelling process was repeated.

Niche overlap and niche divergence tests ran with the packages "ENMTOOLS" (Warren et al., 2010) and "HUMBOLDT" (Brown & Carnaval, 2019). The hypothesis that compared niches are more similar than expected by chance was evaluated with 100 replicates and rejected according to the significance of the test (p < .05). ENMtools estimates niche similarity on the geographical space (G-space), since environmental information is derived from geographical distribution, and was used to measure Schoener's D setting D < 0.33, 0.33 > D < 0.66 and D > 0.66 as evidence of low, moderate and high niche overlap, respectively. We used this metric to group subsets with significantly high niche similarity. The second package projects geographical on environmental space (E-space), explicitly incorporating the spatial distribution of environments or just analogous environments. We followed the methodology described in Brown and Carnaval (2019) to run the "humboldt" Niche Overlap Test (NOT) on all available E-space, that is the total accessible environmental space represented within the geographical distribution of each major phylogroup. Then, the Niche Divergence Test (NDT) was run on analogous E-space, which is the portion of accessible E-space shared by these phylogroups. The "humboldt" workflow allows the estimation of how equivalent (or not) the occupied niches are given a common environmental background and whether low niche equivalence further reflects niche divergence.

#### 3 | RESULTS

#### 3.1 | Population clustering and admixture

STRUCTURE revealed two distinct clusters with minimal admixture  $(Q \ge 95\%)$ , except specimen 1998 with Q = 90%): one corresponding to the northwestern Balkan Peninsula ("North Balkan Clade," hereafter NBC) and the other to the southeastern Balkan Peninsula ("South Balkan Clade," hereafter SBC) (Figure 2a and Figure S2).

The NBC clustered populations from Croatia, Montenegro, north Albania, northwest North Macedonia, Romania and Serbia. Within this group (Figure 2b,c) we detected three subclusters, splitting (a) populations from Croatia (Northwest subclade–NW), - MOLECULAR ECOLOGY -W

(b) Montenegro and Albanian populations from the Montenegro-Albanian borders (Montenegro subclade—MO), and (c) Albanian populations sampled further south, together with North Macedonia, Serbia and Romania (Northeast subclade—NE). The Serbian specimens showed some admixture (Q=85%) between MO and NE; all others had Q>95%.

The SBC clustered the southeastern Balkan populations and Georgia (Asia). Subsequent analysis ran twice including and removing the Georgian specimen. In both cases, we retrieved five subclusters within the Balkan region and several admixed individuals (Figure 2b,c and Figure S2). Populations from the Cyclades islands and Peloponnesos (with the exception of samples 423 and 568 from northeastern Peloponnesos) formed two independent, genetically "pure" subclusters, namely Cyclades (CY) and Peloponnesos (PE). The Balkan mainland split into three additional subclusters: one west of Pindos, including populations from south Albania and northwest Greece (Southwest subclade-SW); another east of Pindos, including southeast Greece. Evvoia Island and south North Macedonia (South subclade-S); and an eastern clade including Bulgaria and northeast Greece (East subclade-E). Admixed individuals were detected in geographical areas of contact between these subclusters. The sample from Georgia was either placed in a sixth subcluster (Caucasus subclade-CA), or was part of the South, with a high percentage of admixture with the East (Q = 28%).

# 3.2 | Phylogenomic trees and estimated divergence times

sNAPP runs on a "no gene flow" assumption, accommodating incomplete lineage sorting (ILS) (Bryant et al., 2012). The admixed specimen from the Caucasus was thus included in our final sNAPP analysis,



**FIGURE 2** Hierarchical population clustering with STRUCTURE. (a) Analysis of all individuals of the Vipera ammodytes species-complex; each bar represents one individual. Bars are coloured according to the optimal number of clusters (K=2) and the proportion of admixture for admixed individuals (Q; given at scale). (b) Analysis repeated for each of the two main clusters, the North Balkan Clade (NBC; left) including three clusters (K=3), and the South Balkan Clade (SBC; right), including five clusters (K=5) and the individual from Georgia, Caucasus, which may represent a sixth cluster (see also Figure S2). In correspondence to Figure 1 and for each individual, sample code is given in (a) as a number above the bar and the respective country code or geographical area of collection is also given in (b) below the bar. (c) The same colours as in (b) are used to map each cluster and each individual. Individuals are represented by pies showing the respective membership percentage (% Q). Clusters are: Montenegro (MO), Northeast (NE), Northwest (NW), Southwest (SW), Cyclades (CY), Peloponnesos (PE), South (S), East (E) and the Caucasus region (CA). A black dashed line shows the approximate geographical separation between NBC and SBC. [Colour figure can be viewed at wileyonlinelibrary.com]

since it probably represents a distinct genetic cluster (Figure 2b and Figure S2), and it is reasonable to assume lack of any current gene flow between Balkan and Caucasian vipers. All other admixed specimens were excluded. Different levels of missing data (uSNPS-100 and uSNPs-75) returned almost identical results in terms of topology, nodal statistical support and estimated times; we present the results from the no-missing-data analysis.

The time-calibrated species-tree shows a clear separation between the NBC and the SBC (Figure 3a). The divergence between them is estimated at 5.1Ma (4.1–6.3Ma). In NBC, NW splits first, ~2.0Ma (1.5–2.5Ma), while the sister-subclades NE and MO began to diverge some 1.2Ma (0.9–1.6Ma). In SBC, the sister-subclades E and CA split from the others some 3.0Ma (2.3–3.6Ma). The divergence between them is dated at 2.5Ma (1.9–3.1Ma). The remaining subclades are monophyletic and their divergence at 2.2Ma (1.7– 2.7Ma) represents a polytomy between PE, SW and all other subclades. Finally, the Cycladian subclade CY splits from S at ~1.7Ma (1.3–2.1Ma).

The two SVDQUARTETS phylogenomic trees have similar topologies and support values, but the uSNPs-100 presented less resolved relationships, due to the smaller number of analysed SNPs. Both data sets identify the same two major assemblages, NBC and SBC (Figure 3b). In NBC, subclades NW, MO and NE form a polytomy. In SBC, there is also a polytomy between SW, E+CA, PE and the remaining subclades. Within the latter unit, populations of southeast Greece (South subclade—S) split into two: South-2 (S2) from the northern part of east Greece splits first, while South-1 (S1) from the southern parts is sister to CY with high statistical support.

The ML tree of individuals, based on the concatenated data set, also confirms the occurrence of NBC and SBC (Figure 3b). In NBC, we find a polytomy between NW, MO and NE. In SBC, there is also a polytomy between SW, E+CA and the remaining subclades. In the latter assemblage, the Peloponnesian subclade PE splits first, with all others being monophyletic. Then S2 from the northern part of east Greece splits, while S1 from the southern part is sister to CY with absolute statistical support. Within CY, populations from the north and south Cyclades islands form two respective subclades.

#### 3.3 | Introgression between clades

The ML population-tree from TREEMIX (Figure 3c) presents the same topology as the SNAPP species-tree. Although adding migration edges m improves the tree, 99.9% of the variance is explained with the first three migration edges, while likelihood scores and explained variance do not significantly improve when m > 3. The highest  $\Delta m$  according to the Evanno method corresponds to m = 1 (Figure S3). The first migration edge is from East to S2, the second from S2 to SW, and the third from MO to NE (Figure 3c).

The f3 test among all possible triplets of populations (360 in total) returned positive values for f3 and z scores, with the single exception (f3 = -0.0014, z = -2.86) of S2 that does not form a simple tree with East and S1, indicating that it is admixed between the

two. The f4 test among all possible quartets returned three cases of significant deviation from "treeness" (Table S3). All three indicate introgression between East and S2.

# 3.4 | Mitochondrial gene-tree and alternative hypotheses

The mitochondrial gene-tree and the MPTP analyses revealed 11 strongly supported mitoclades (Figure 4a and Figure S4). Ten of them had been reported before (Čubrić et al., 2019; Ursenbacher et al., 2008) and are confirmed here with more sampling localities that increase their known geographical distribution (Figure 4b). An eleventh one is reported for the first time from the North-Eastern Peloponnese (NEP), as sister to the mitoclade distributed in the southeast Balkan mainland (South 1+2).

Most of the relationships found in the mtDNA phylogeny are unresolved, having weak to moderate support. Most of the mitoclusters and clades coincide with those identified by the genomic analyses. There are, however, some major or minor differences; for example, the three NBC subclades, although situated at the base of the phylogenetic tree, do not form a monophyletic group. Therefore, we tested the following alternative relationships: (i) all *Vipera ammodytes* from the northern and the southern Balkan Peninsula (including Asia) to be reciprocally monophyletic; (ii) East and Caucasus to be sister-clades; (iii) South 1+2 to be divided into two monophyletic units, situated respectively at the south (S1) and the north (S2) of its distribution; (iv) Cyclades to be sister to South 1+2, or (v) Cyclades to be sister to S1. The first alternative topology test showed that a maternal phylogeny with two major monophyletic groups, NBC and SBC, is not rejected (Table S4).

The south Balkan and Asia populations form eight subclades with mostly unresolved relationships. They represent a polytomy between SW, PE, CY and a monophyletic group that, in turn, includes a polytomy between CA, Turkey (TR), and the group of East and South 1+2, the last being sister to NEP. The alternative topology tests do not reject the monophyly of East and CA, or the split of South 1+2 into S1 and S2. They reject the sister-relationship between CY and South 1+2 or S1. Similarly to the phylogenomic trees, the populations from the north and central Cyclades islands form two respective subclades, while, interestingly, a single sample from the neighbouring part of Evvoia is placed as sister to the two Cyclades subclades. All other samples from Evvoia are nested within South 1+2.

#### 3.5 | Niche modelling and niche divergence

Model performance was high in all cases, with average test AUC ranging from  $0.85\pm0.005$  (South) to  $0.90\pm0.012$  (Asia). The bioclimatic variables contributing more to each model were associated with precipitation for the North (bio12, bio17; accumulated relative contribution of 60%) and temperature during months of harsh



FIGURE 3 (a) Genomic species-tree derived from the Bayesian coalescence analysis of the uSNP-100 data set (unlinked SNPs with no missing data) including only nonadmixed individuals. Analysis was run with sNAPP and was transformed into a time-calibrated chronogram following Stange et al. (2018). *Vipera berus* was used to root the tree (not shown) and the divergence time between *V. berus* and *V. ammodytes* was used as the calibration point. Mean divergence-time estimates (in million years) are given for each node and grey bars represent the 95% highest posterior density (HPD). The statistical support of the node is absolute when marked with a black dot or almost absolute (SNAPP posterior probability p = .98) if marked with a white dot. Geological periods of the Cenozoic Era and their respective range are presented in a scale below the chronogram; MSC, Messinian Salinity Crisis. (b) ML phylogenomic tree based on the concatenated ddRAD loci data set of all individuals, produced with IQ-TREE. A similar topology was derived from the svDQUARTETS analysis on the uSNP-75 data set (unlinked SNPs with 25% of missing data) (tree not shown). Numbers above branches (shown only when >50) give the respective nodal support estimated as SH-aLRTs/ultrafast bootstraps/bootstrap values estimated for the same node inferred by svDQUARTETS. The major phylogenetic groups are marked and colour-shaded in correspondence to Figure 3 and the respective branches of the chronogram. Note that the S cluster of Figure 4 is split here in S1 and S2. (c) TREEMIX admixture graph with three migration events (m). The migration weight and the directionality of the inferred introgression are indicated by the coloured arrows. The graded bar on the left approximates colours to values of migration weight as the fraction of ancestry derived from the migration source, so that the warmer colour (dark red) indicates an ancestry of 50%. The scale below measures the genetic drift from the ancestral to the extant populations. [Colour figure can be viewed at

climatic conditions for the remaining groups, for example temperature ranges (bio2, bio15; 85%) for Cyclades, and temperature during the driest quarter for South (bio4, bio9; 70%) or the wettest quarter for Asia (bio4, bio8, bio15; 72%). Topography had a lesser contribution (≤10%) in all cases. With the exception of Cyclades, most of the response curves showed similar trends for the three other groups (detailed results on all models are presented in Figure S5 and Table S5).

The ENMTOOLS estimation of niche identity (Table S6) returned low to moderate D values in most pairwise comparisons (0.07– 0.46). Significantly low values were found between Cyclades and any of the remaining groups, suggesting lack of niche overlap. Asia and South showed a high value (D=0.67), niche similarity was not rejected and thus we repeated SDM on South+Asia, finding low niche similarity between this new group and either Cyclades or the North. According to the new model for South+Asia, presence probability is again affected by temperature (bio4, bio6, bio9; 63%). The respective habitat suitability (HS) maps (Figure 5a,b) showed a spatial coverage (where North/South+Asia might occur) very similar to the actual distributions of the NBC and SBC clades. Suitable habitat for each group was mostly segregated.

NOT and NDT tests with "HUMBODLT" were run on the North and South, alone and including Asia. Due to the small area of distribution of the Cycladian vipers and the relatively low number of available presence-points, tests with Cyclades were not performed. NOT gave significant Equivalence and Background statistics (Table S6), suggesting nonequivalent niches between North and South, while NDT estimates were not significant, rejecting the hypothesis of divergent evolution. When only analogous environmental space is evaluated (NDT), the area occupied by North and South+Asia overlaps (Figure 5c). Both NOT and NDT returned nonsignificant Equivalence statistics with significant Background statistics, giving strong evidence for equivalent niches between North and South+Asia (Figure S6).

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# 4.1.2 A similar pattern of geographically structured genetic diversity is observed in the SBC. Based on the genome-wide SNPs (Figure 3b), this clade includes four phylogeographical groups defined by major

biogeographical barriers, which are the Pindos range along the mainland, and the sea-barriers separating Peloponnesos and the Cyclades island group from the continental parts of the Balkan Peninsula (Figure 1 and Figure S1). The respective genomic groups are (i) the Southwest (SW) situated west of Pindos, (ii) the South (S1+S2) and Cyclades (CY) on the east side of the same mountain system, (iii) the Peloponnesian endemic (PE), and (iv) the East (E) and Caucasus (CA) found further east in the Balkan Peninsula and into Asia. The consensus among all phylogenomic analyses is that these four groups form a polytomy, as a result of a synchronous radiation that took place sometime around the Plio-Pleistocene boundary or in the early Pleistocene (Figure 2a).

Pindos is an important biogeographical barrier for the diversification within the SBC, with distinct genomic and mitochondrial clusters occurring along its west and east side (Figures 2c, 3b and 4b). Many reptile and amphibian species are restricted totally or mostly on one or the other side (e.g., Psonis et al., 2018; Strachinis et al., 2021), and several phylogeographical studies have found intraspecific lineages distributed in allopatry (e.g., Kindler et al., 2013; Kornilios et al., 2019; Marzahn et al., 2016; Thanou et al., 2020), suggesting the role of subrefugia situated on each side.

Peloponnesos, the most species-rich region for reptiles in the entire European continent (Sillero et al., 2014), hosts endemic genera, species and phylogenetic lineages (Jablonski et al., 2016; Kornilios et al., 2019; Spilani et al., 2019; Strachinis et al., 2021; Thanou et al., 2021). Peloponnesos was connected to the current

#### | DISCUSSION 4

#### 4.1 Geoclimatic drivers of speciation

#### 4.1.1 MSC-driven early divergence

The genomic SNP analyses (Figures 2 and 3) provide strong evidence for the existence of two highly divergent evolutionary lineages within the Vipera ammodytes complex: the NBC is distributed in the northwest and the SBC in the southeast of the Balkan Peninsula, expanding into Asia as far as the Caucasus. This north-south differentiation is not unequivocally seen in the mitochondrial gene-tree (Figure 4), but according to the alternative topology tests it is probably also true for the maternal phylogeny (Table S4).

The divergence between NBC and SBC dates back to the Miocene/Pliocene boundary (Figure 3a), and is most probably linked to the Messinian Salinity Crisis (MSC), a usual suspect of major diversification events in the region, especially for amphibians and reptiles (e.g., Gvoždík et al., 2013; Kornilios et al., 2012; Kyriazi et al., 2013; Sindaco et al., 2014; Skourtanioti et al., 2016; Wielstra et al., 2010). Starting at 5.96 Ma, the Mediterranean basin became progressively isolated from the Atlantic Ocean, triggering a dramatic sea-level lowering that formed land bridges between islands and mainland (Krijgsman et al., 2010 and references therein). Sea-level change and large-scale evaporation led to major, localized climatic impacts (Ivanovic et al., 2014 and references therein), a global climate change from hotter and wetter to colder and more arid environments (Fortelius et al., 2002; García-Alix et al., 2008), and a subsequent vegetation and habitat turnover (Cerling et al., 1997). The MSC ended with the reflooding of the Mediterranean from the Atlantic at the beginning of the Pliocene (5.33 Ma) (Hsü et al., 1977), which is assumed to have triggered allopatric speciation due to local extinctions of populations and the survival of differentiated ancient genetic stocks. The spatiotemporal divergence pattern of the nosehorned viper's ancestor is remarkably similar to that observed in the lacertid lizard species-group of Podarcis tauricus (Psonis et al., 2018). Both the time and place of these two divergences coincide perfectly: Podarcis melisellensis, which has a similar distribution to NBC, split some 5.5 Ma from the other species of the group, which are distributed in the south and east of the Balkan Peninsula, similarly to the SBC.

The genetic diversification of the NBC and SBC clades is not accompanied by significant ecological divergence, according to our results (with the possible exception of the Cycladian lineage). A recent study on the V. aspis species-complex from the west side of the Mediterranean also highlighted the importance of refugia and subrefugia and the role of local extinctions, and showed that climate adaptation played a secondary role in lineage diversification (Martínez-Freiría et al., 2020). Here, the equivalency and similarity tests (Table S6) show different availability of suitable climatic conditions within the respective range of NBC and SBC. Niche differences identify two climatically distinct regions, but the similar selection patterns (Figure 5c and Figure S6) suggest that vipers of both clades

inhabit analogous environments. During the Pleistocenic glacial periods, ice caps became established on the Dinarides along the Adriatic Sea, blocking the inland penetration of humid Atlantic air masses and promoting arid conditions in the Balkan interior. Nevertheless, the Dalmatic coasts and the southern Balkans showed milder conditions and reduced expression of glacial-interglacial climate variability (Fitzsimmons et al., 2012; Hughes et al., 2010). These may be the refugial areas for the NBC and SBC, respectively, which provided pockets of similar and relatively stable climatic conditions.

#### **Plio-Pleistocene radiation**

Both biparentally (SNPs) and maternally (mtDNA) inherited markers recognize three geographically defined subclades within the NBC (Figures 2-4); their diversification started at the beginning of the Pleistocene possibly with the split of NW from the other two (MO and NE) (Figure 3a). The Montenegrin subclade is geographically confined, while NW and NE are respectively expanding to its northwest and northeast (Figure 4b). This phylogeographical structure and the time period of diversification within the NBC are concordant with the "refugia within refugia" model, in which recurrent cycles of population expansion and contraction are driven by the Pleistocene climatic oscillations (Ursenbacher et al., 2008).



FIGURE 4 (a) The mitochondrial gene tree, from the maximum-likelihood (ML) analysis with IQ-TREE. The outgroup is not shown (see text for details) and the major phylogenetic clades are collapsed. Nodal support gives the values of SH-aLRTs/ultrafast bootstraps/standard bootstraps and are shown only when >50. Absolute support is marked with a black dot. The sample codes, country codes and/or the general geographical location of the individuals included in each clade are given. (b) Map of the approximate distribution of each mitoclade, using the same colour-coding and in correspondence to Figures 2 and 3. These are Montenegro (MO), Northeast (NE), Northwest (NW), Southwest (SW), Cyclades (CY), Peloponnesos (PE), Northeast Peloponnesos (NEP), South (S), East (E), Turkey (TR) and the Caucasus region (CA). Note that clade S includes samples of S1 and S2 of Figure 3. A black dashed line shows the approximate geographical separation between the North Balkan Clade (NBC) and the South Balkan Clade (SBC). Dashed grey lines show the distribution area of the respective mitochondrial clade, according to previous knowledge (Čubrić et al., 2019; Ursenbacher et al., 2008). [Colour figure can be viewed at wileyonlinelibrary.com]



FIGURE 5 Prediction map of the area of habitat suitability (HS) for the Vipera ammodytes species-complex based on species distributional models (SDM) built with MAXENT. Models were built using presence-points representing (a) the North Balkan area of distribution (North), and (b) the South Balkans, including records from Asia (South+Asia). For details on models, variables and their importance for model-building, see Figure S5 and Table S5. The graded bar on the left approximates colours to values of HS, so that the warmer colour (dark red) indicates HS=1. (c) Density plots of the analogous environmental space (E-space) occupied by North (blue) and South+Asia (red), estimated in "HUMBOLT." Niches for both groups are shown in a single plot, where higher densities are shown with darker colours and lines represent the kernel density isopleths from 1% to 100% (periphery to centre). On the top and right side, histogram density plots for each of the two first PCs are shown; each E-space is displayed in the respective colour (blue and red) and niche overlap with the combination of the two colours (purple). [Colour figure can be viewed at wileyonlinelibrary.com]

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Greek mainland until the end of the Miocene and disconnected with a wide sea-barrier at the beginning of the Pliocene (5–3.5 Ma) (Dermitzakis, 1990). During the Pleistocene glacial/interglacial cycles, it acted as a major refugium with several subrefugia (Thanou et al., 2014 and references therein), as sea-level changes resulted in new connections with the north and new dispersals and subsequent isolations. The highly divergent Peloponnesian vipers (Figure 3a,b, and Figure S4) could have colonized Peloponnesos during sea-level lows, through emerging land-bridges, and then have become isolated during sea-level highs related to the Pleistocene climatic oscillations. Alternatively, their split could be attributed to vicariant isolation after the disconnection of Peloponnesos at the Plio-Pleistocene boundary.

In the eastern part of the Balkans, clade E, and its sister subclade representing the Asian part of the viper's distribution (CA) diverged from the other SBC groups at the late Pliocene or the Plio-Pleistocene boundary. During that time, vipers and other reptiles survived in isolated areas of the eastern Balkans, such as between the Balkanides and the Macedonian-Thracian massif (Figure S1; see Jablonski et al., 2016). Later they dispersed eastwards into Asia, probably following a route along the Black Sea coast and reaching the Caucasus Mountains, as suggested by Ursenbacher et al. (2008).

Within both the NBC and SBC, divergent subclades can be geographically referred to a putative (sub)refugium that, during glacial periods, was disconnected from adjacent regions by extended glaciers (Figure S1). For example, MO was confined among past glaciers of the Orjen, Durmitor and Prokletije Mountains (Hughes et al., 2011), the glacier of Mount Olympos Mt (Smith et al., 2006) lay between the distributions of S1 and S2, and the glacier of Mount Chelmos (Pope et al., 2017) separated NEP from the other Peloponnesian populations. Phylogeographical analyses complemented with past and present niche projections are useful tools for the delineation of the multiple subrefugia integrating the Balkans (Martínez-Freiría et al., 2017; Mezzasalma et al., 2018; Strachinis et al., 2021; Wielstra et al., 2013), and the nose-horned vipers seem to be an excellent study system for such analyses.

#### 4.1.3 | Overseas colonization of Cyclades

All results based on the genome-wide loci show that the populations east of Pindos (S) are sister to the insular populations of the Cyclades archipelago (CY), indicating that the central Aegean islands were colonized from this part of the Balkan Peninsula. These islands once formed a continuous landmass, the Cyclades Plateau (Figure S1), which was connected to the Balkan mainland (present southeast Greece, including Evvoia). At ~3.5 Ma, the land bridge between them was submerged and never re-established (Anastasakis et al., 2006). This vicariant event has led to Cycladian endemic lineages in several reptiles (Kornilios et al., 2019; Kyriazi et al., 2013; Thanou et al., 2020).

The colonization of the Cyclades island-group is dated here to 1.7 Ma (Figure 3), contrary to previous much older estimates of

3.5 Ma (Ursenbacher et al., 2008). Our estimation was based on multiple unlinked genomic loci and a coalescence approach, while the earlier dating used a mitochondrial gene-tree, where the Cyclades and Peloponnesos were found as sister clades. This sister-clade relationship, which greatly affected the previously estimated divergence times, is not seen in our mtDNA analyses (Figure 4 and Figure S4) and was probably the result of long-branch attraction (see Kornilios, 2017) between those two long mitoclades. In fact, our mtDNA gene-tree reveals a relationship between Cyclades and a single sample from the southern part of Evvoia (sample 555; Figure 4), confirming the affinity between the Aegean insular populations and the neighbouring mainland.

The divergence between CY and S (South-1+South-2) occurred during the mid-leistocene (Figure 3), when the islands had already undergone prolonged isolation from the mainland. This implies overseas dispersal, which would have been more probable during glacial periods, when sea level in the Aegean was much lower than at present (Lambeck, 1996). During the Pleistocene, the Cyclades Plateau began breaking into two island-groups (Kapsimalis et al., 2009): one north, including present-day Syros and Tinos, and one south, including Antiparos, Naxos and Ios. Populations from these islands form two respective subclades in both the genomic and mitochondrial phylogenies (Figure 3b and Figure S4). Overseas dispersals to Aegean islands during glacial periods and the gradual fragmentation into the current Cyclades islands have shaped the diversity of several reptiles and amphibians (Dufresnes et al., 2018; Kornilios et al., 2019; Kyriazi et al., 2013; Skourtanioti et al., 2016; Thanou et al., 2020).

# 4.2 | Dispersals, secondary contacts and hybrid zones

As already highlighted, the biogeographical patterns and diversification history of the nose-horned viper are linked to (i) the Plio-Pleistocene geoclimatic changes that led to cycles of allopatric isolations in refugia and subrefugia, and (ii) the occurrence of biogeographical barriers or corridors in the Balkan Peninsula. Our analyses of thousands of genome-wide SNPs provide evidence of subsequent expansions from these refugia, secondary contacts between diverged lineages and the formation of multiple hybrid zones.

Based on the samples analysed so far, NBC and SBC do not appear to be sympatric, while the smallest observed distance between them is ~50 km in Serbia (samples DQ186513 and DQ186483) and 70 km in North Macedonia (samples 1629 and 1998; Figure 1). Only one sample (1998) exhibits some genetic admixture between these clusters (90% and 10%, respectively) indicating very low, if any, hybridization between them, but TREEMIX did not detect current or past introgression. The diversification between these two major evolutionary lineages lies at the oldest end of the speciation continuum for the *V.ammodytes* complex, with strong evidence that it represents a case of complete speciation.

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At the more recent end of this speciation continuum, multiple incomplete speciation events occurred in the late Pliocene and the Pleistocene within NBC and SBC. Introgression and hybridization is observed in almost every secondary contact zone that could possibly have formed in the Balkan region. Within NBC, STRUCTURE and TREEMIX detect gene flow from MO to NE (Figure 3c). The NW subclade was represented in our genomic data set with only one sample from an area quite far from its expected contact zone with the other two subclades. Recently, Čubrić et al. (2019) reported syntopic vipers belonging to NW and NE mitoclades from two locations in northern Serbia. Hence, although we did not find evidence of genetic admixture between NW and the other NBC subclades, this may occur.

The phylogeographical and genetic patterns within SBC are more complex. The SW and S subclades, that respectively differentiated on each side of Pindos, form two contact zones, one in the north and one in the south edge of the mountain range, where extensive genetic admixture was observed (Figure 2b) and TREEMIX detected migration from S to SW (Figure 3c). This resembles an incomplete "ring-species" diversification pattern. When populations diverge around a geographical feature, such as a mountain, they can form a ring-like distribution whereby neighbouring populations can interbreed but populations that meet at the terminus of the respective ranges are reproductively isolated forming a ring-species (Irwin & Wake, 2016). Here, the diverged populations meet at two points, north and south of the geographical barrier as a result of secondary expansions, but reproductive isolation is incomplete and the ring-species divergence is ephemeral, as shown in other cases of reptiles with a Pleistocene diversification history (Bouzid et al., 2022). There is also evidence of genomic introgression directed from an ancestor of the E subclade to S (Figure 3c), suggesting west and south expansion from an east Balkan refugium. Ultimately, in the north of Pindos we find individuals that are genetically admixed between all three neighbouring subclades E, S and SW.

Finally, subclade S forms a fourth hybrid zone at the northeast edge of Peloponnesos, suggesting that it has either expanded into Peloponnesos or survived in a refugium there and then interbred with the populations of the endemic subclade PE (Figure 2b). All Peloponnesian individuals belong to the endemic mitochondrial subclade (PE), with the exception of sample 568 from the northeast edge that is not related to the Peloponnesian endemics and forms its own mitoclade (NEP) (Figure 4 and Figure S4). This is an independent indication of dispersal into Peloponnesos and, since this individual is quite differentiated from all others of southeast Greece, this dispersal is probably not recent. Not surprisingly, this sample is genetically admixed between S and PE, according to the genome-wide SNPs. In northeastern Peloponnesos, we find both admixed and genomically "pure" S samples with PE mitogenomes, as evidence of both nuclear and mitochondrial introgression occurring in this hybrid zone.

#### 4.3 | Resolving the mitonuclear discordance

Most inconsistencies observed between the genomic and mitochondrial phylogenies are clarified after performing the alternative topology tests. The two phylogenies are only truly conflicting in one relationship: in the genomic trees (Figure 3) the populations east of Pindos are sister to the Cyclades islands but in the mitochondrial gene-tree (Figure 4) the same populations are sister to the east Balkans. Genomic analyses differentiate the S populations into two subclades, S2 in the north and S1 in the south and, although all samples of the S mitoclade exhibit low mitochondrial diversity, the alternative topology test did not reject this possibility for the mitochondrial genome. The simplest explanation is that while the genomic SNPs can detect a north-south geographical genetic structure the mtDNA cannot, due to ILS.

A second explanation, on its own or in combination, may be inferred by the genetic admixture found between East and S2 (Figures 2 and 3c). Although admixed individuals were not included in the ML and svDQUARTETS analyses, it is possible that the nonadmixedaccording to STRUCTURE-northern individuals of the S clade still bear some East genomic DNA. This would result in their differentiation (S2) from the more southerly populations (S1) and the formation of a progressive cline with "pure" E and S populations at the northeast and southeast edges, respectively. Moreover, a single sample (555) from south Evvoia clusters within S1 (Figure 2) but is not nested within the respective "South 1+2" mitoclade (Figure 4). Instead, it creates its own branch, sister to the Cyclades, implying that these southerly regions retain an ancestral mitogenome. Otherwise, this mitochondrial relationship can only be explained by a reverse overseas recolonization of south Evvoia from the Cyclades islands, which is far less likely, since island-to-mainland dispersal is considered to be a rare phenomenon (Nicholson et al., 2005).

To sum up, we are able to propose a biogeographical and evolutionary scenario that fits all results and explains all mitonuclear discordances. This scenario, schematically depicted in Figure 6, proposes extensive genetic admixture through hybridization and introgression, and the extended replacement of the ancestral mitogenome in most of the South populations by the mitogenome of the East, as the latter expanded in a southwest direction. Consequently, a zone of introgressive hybridization with asymmetric mtDNA capture developed east of Pindos, with genomically "pure" populations occurring at the two edges of the cline. At the northeast edge, populations hold the ancestral East mitogenome, while slightly differentiated versions (S and NEP; Figure 4) replaced the ancestral south mitogenome in a southwards progression; the latter is currently maintained only in the southernmost edge, namely south Evvoia. Conclusively, the "True South" corresponds to the genomic subcluster S (S1+S2) and the mitoclade where sample 555 is placed, and the "True East" corresponds to the genomic subcluster E and the southeast mitogenome (E+S+NEP). This explains all mitonuclear conflicts: in both phylogenies Cyclades is sister to the "True South," while the Asian phylogroup is sister to the "True East."

Introgressive hybridization with asymmetric mtDNA capture (Bonnet et al., 2017) has also been proposed as the mechanism behind the mitonuclear discordant patterns in the only other study that utilized genome-wide SNPs on *Vipera* (Zinenko et al., 2016). A similar pattern of extensive mitochondrial capture among mainland



FIGURE 6 Schematic representation of our scenario proposed to explain the phylogenetic relationships and population histories within the South Balkan Clade (SBC) of the Vipera ammodytes species complex. (a) Approximately 1.5 Ma, the major phylogenetic groups had separated. These were respectively distributed in Peloponnesos (PE), west of the Pindos mountain range (SW), east of Pindos (True S) throughout the Cyclades Islands (CY), the Eastern Balkans (True E), and further east into Asia (TR+CA). True S and CY were sisterclades, as well as True E and TR+CA. (b) The climatic cycles of the Pleistocene caused respective fluctuations in population size and the divergence of isolated populations, for example the split between western Turkey (TR) and the Caucasus region (CA) and the split of CY into two assemblages, the north and the south Cyclades islands. During periods of population expansion, mainland populations reached secondary contact, resulting in genomic introgression (red arrow) from the True E into the True S. (c) Through repeated expansion cycles, a cline of genomic hybridization between True E and True S occurred east of Pindos, with pure populations of the True E and True S genomes, respectively, in the northern and southern edge of this cline. This also led to the progressive replacement of the ancestral True S mitogenome by the True E mitogenome (black arrow). The former was "pushed" southwards (red lines), while the latter reached into the northeastern part of Peloponnesos. Secondary contact zones were also formed at the north and south edge of Pindos between SW and the respective population east of Pindos. (d) Currently, the True S mitogenome is retained only in the southernmost edge of its former distribution (presenting the same sister-clade relationship with the Cycladian populations), while the True E ancestral populations evolved into the E, S2, S1 and NEP. Hybridization zones are established north and south of Pindos, forming an ephemeral ring-species (dashed line around Pindos) with significant genomic introgression from S2 to SW (yellow arrow). [Colour figure can be viewed at wileyonlinelibrary.com]

populations, with an old mitogenome retained in southern Evvoia, was proposed in the phylogeographical study of the four-lined snake (Thanou et al., 2020). Mitochondrial capture is now considered to be a quite common evolutionary process (Bonnet et al., 2017) and it has been repeatedly reported in amphibians and reptiles (Thanou et al., 2020 and references therein). Our evidence supports that it has probably also affected the nose-horned viper's evolutionary history.

#### 4.4 | Species within the species-complex

Presently, four taxa are accepted within V. ammodytes: V. a. ammodytes (Linnaeus, 1758), V. a. meridionalis (Boulenger, 1903), V. a. montandoni (Boulenger, 1904) and V. a. transcaucasiana (Boulenger, 1913), with transcaucasiana treated either as subspecies (e.g., McDiarmid et al., 1999; Tomović, 2006; Ursenbacher et al., 2008) or as full species (e.g., Freitas et al., 2020; Mallow et al., 2003; Nilson et al., 1999; Šmíd & Tolley, 2019). Their geographical distribution is presented in the maps of Figure S7, based on three studies (Golay et al., 1993; Heckes et al., 2005; Tomović, 2006). All three agree in the morphological recognition and geographical distribution of *ammodytes*. Within the southern Balkans, Golay et al. (1993), contrary to Heckes et al. (2005), did not distinguish *montandoni* from *meridionalis*, while Tomović (2006) showed a northwest to southeast morphological cline. When the respective morphological taxa are mapped on our phylogenies (Figure S8) it becomes apparent that genomic results favour the Heckes et al. (2005) scheme, while following Golay et al. (1993) or Tomović (2006) returns several paraphylies.

Ursenbacher et al. (2008) noted the inconsistency between phylogenetic relationships and current taxonomy. Our genomic and mtDNA phylogenies recognize two largely allopatric assemblages with an old divergence time, the NBC and SBC, which exhibit minimal or no gene flow and a clear morphological differentiation: all samples of the NBC belong to the *ammodytes* morphotype, while all other morphs (*meridionalis, montandoni, transcaucasiana*) are nested in the SBC. Coupled with the high degree of inferred reproductive isolation, there is good evidence that NBC and SBC represent different species, namely V. ammodytes and V. meridionalis. By contrast, the extensive gene flow, multiple hybrid zones and low genetic differentiation among the subclades within each of these major clades point to limited, if any, reproductive isolation and a pattern of incomplete speciation. For transcaucasiana, our genomic data further support that it does not merit species-level status (see Tomović, 2006; Ursenbacher et al., 2008). Regarding the insular Cycladian cluster, it is less genetically divergent and more recent compared to the other intraspecific subclades within the SBC. Cycladian vipers exhibit a profoundly smaller size among other differentiating morphological traits (P. Kornilios, E. Thanou, unpublished data; Cattaneo, 2010; Itescu et al., 2018) and a different ecological profile according to our SDMs. However, these ecomorphological characteristics are probably the result of local adaptation to the Aegean insular environment (climate, vegetation, prey, predators, etc.) triggering island dwarfism, rather than evidence supporting species status.

#### AUTHOR CONTRIBUTIONS

PK designed the study. All authors contributed with resources, sample and data curation. ET and PK performed DNA data generation, data processing and analyses. ET wrote the original draft with contributions, reviewing and editing from all authors.

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#### CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

#### DATA AVAILABILITY STATEMENT

Processed ddRADseq data files and input files for analyses included in the paper are hosted on Dryad (https://doi.org/10.5061/ dryad.866t1g1vp). Mitochondrial *cytb* data are deposited in the NCBI Nucleotide Database (Accession nos. OP973940–OP974015). Related metadata can be downloaded from GBIF (2021) (https:// doi.org/10.15468/dl.58wq2v; including georeferenced points in decimal degrees) and additional georeferenced data of our own unpublished sources may be shared upon request to the correspond-

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#### SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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# **MOLECULAR ECOLOGY**

#### Supplemental Information for:

## Genome-wide SNPs reveal recurrent waves of speciation in niche-pockets, in Europe's most venomous snake

Evanthia Thanou, Daniel Jablonski, Panagiotis Kornilios

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#### **Supplementary Figures**



**Figure S1.** Map of the major geographical features that have presumably acted as refugia-within-refugia in the Balkan Peninsula and are discussed in the text (roughly re-drawn after Jablonski et al., 2016). The dashed white lines correspond to the major mountain systems in the area and the white line shows the approximate position of the Pindos Mt. Range. Numbers 1-5 point to the approximate position of the most extended and possibly oldest Balkan glaciers: (1) Orjen Mt., (2) Durmitor Mt., (3) Prokletije Mt., (4) Olympus Mt. and (5) Chelmos Mt (Smith et al., 2006; Hughes, 2007; Hughes, 2010; Hughes et al., 2011; Pope et al., 2015; Hughes & Woodward, 2016). Black outlines roughly depict the extent of the great steppe formed in the Danube Basin during glacial periods (by Kovać et al., 2017) and the Cyclades Plateau (CP) after its isolation from the mainland at approximately 3.5 Mya (by Kapsimalis et al., 2009).



**Figure S2.** Results from the STRUCTURE analyses. Plots of Evanno's Delta K (Evanno et al., 2005) to the number of assumed population clusters K for (A) all samples of *Vipera ammodytes*, (B) samples belonging to the North Balkan Clade - NBC (see main text), (C) samples belonging to the South Balkan Clade - SBC (see main text), and (D) samples belonging to SBC after excluding the specimen from Georgia. For C and D we show the respective assignment plots of the individuals included in each analysis. In C, when Georgia is included best number of clusters is K=5 (top), however for K=6 (bottom) Georgia is moved to its own cluster. In D, with Georgia excluded from the analysis, again K=5.



**Figure S3.** Results from the TREEMIX analyses. Plot of the mean likelihood-scores with the respective standard deviation and the explained variance (top) and of Evanno's Delta m (bottom) to the number of assumed migration events m, for m=1-10.



**Figure S4.** The Maximum Likelihood (ML) phylogenetic tree based on the *cytb* dataset, including 142 *V. ammodytes* sequences and 7 outgroup sequences. The eleven major phylogenetic clades retrieved for *V. ammodytes* are colour-shaded. Coding in terminal nodes refer to samples in Figure 1 and Supplementary Table S1. Numbers in branches give the respective nodal support estimated as SH-like approximate likelihood ratio test / ultrafast bootstrap values / standard bootstrap alignments.

0.03























bio4







57



1.0

148

156











SOUTH + ASIA bio2 1.0

0.5

0.0

49











-164

95

-41

230

Figure S5. MaxEnt models built from all the available geo-referenced presence points of Vipera ammodytes (ALL) and for specific subsets of those points, namely the Cyclades Islands, Asia, South Balkans, North Balkans, and for South Balkans including Asia. For details on the parameterization process we followed during model-fitting, the final variables included and the percentage of variable importance, also see Supplementary Material Table S5. In

ALL

-107



pages 6 & 7, we present the respective niche suitability map, and above it, a map depicting the distribution of points included in each final model (left), plots of the Jackknife tests of variable importance (centre), and plots of the receiver operating characteristic (ROC) curve averaged over 100 bootstrap replicates (right). The response curves of the variables with the highest contribution for each model are also shown, in pages 8 & 9.



Figure S6. Results of the "humbolt" NOT and NDT tests for the analogous spaces occupied by "species 1" and "species 2", which represent respectively the presence points of V. anmodytes found in the South Balkans including Asia, and the North Balkans. On the top, we show the respective E-space density plots corresponding to the occupied environments (not species). Black lines show kernel density isopleths (solid line = 0.1, dashed line = 0.5). Frequency of the respective environment in space is colour-coded (white = absent, cold to warm = low to high frequencies). At the bottom left, a single plot comparing the E-space frequencies of the two environments: red colours depict increasingly higher E-space kernel density values for environment 1, blue respective values for environment 2 and white shows areas of identical kernel density values. Grey lines circle the E-space corresponding to environment 1; black lines mark isopleths corresponding to differences between the two E-spaces. Note that the high uncorrected value of the E-space abundance test (0.956) suggests a high overlap among the differences between input environments and the differences between species, thus we corrected the occurrence densities of each species by the prevalence of the environments in their range for Equivalency, Background, and overlap tests (correct.env=T). The results of these analyses (see also Supplementary Material Table S6) are given at the bottom right: Niche Similarity estimates Schoener's D among the two niches, while the second value measures niche similarity only in analogous environmental space. Equivalency plots a one-tailed statistical test of the null hypothesis that niches are equal; the test compares the observed niche similarity between the two species (red diamond) and the overlap of niches built from the repeated resampling of occurrences of species 1 and 2 (blue histogram). The non- significant value states that the two species' datasets are statistically equivalent, thus the null hypothesis that niches are equivalent cannot be rejected. Background Test on the niche similarity in E-space compares the observed niche similarity between the two species (red diamond) to the overlap between species 1 and the random shifting of the spatial distribution of species 2 in geographic space. Then measures how that shift in geography changes occupied E- space and creates a null distribution of available E-space in the habitat of species 2 (blue density curve). This is then respectively measured for species 2. For more details see Brown & Carvanal (2019).



ammodytes meridionalis montandoni transcaucasiana



Figure S7. Distribution maps of the morphological subspecies described within the *Vipera ammodytes* species-complex, according to three taxonomical proposals by Heckes et al. (2005), Golay et al. (1993), and Tomović (2006).



**Figure S8.** Mapping of the morphological subspecies (see Figure S8) on the ddRAD-based species-trees (top) and the mtDNA-based phylogenies (bottom). The major clades are colour-coded to depict subspecies, as proposed by Heckes et al. (2005) in (A) and (D), Golay et al. (1993) in (B) and (E), and Tomović (2006) in (C) and (F).

#### **Supplementary Tables**

**Table S1.** List of specimens used in this study. Specimen working codes and the respective localities of collection are in accordance with Figure 1. Each specimen is taxonomically assigned to one of the currently recognized *V. anmodytes* subspecies or species, following the geographical distribution of each taxon, as proposed by Heckes et al. (2005), Golay et al. (1993) or Tomović (2006). Blank cells denote samples that failed to produce ddRAD or *cytb* data. For the *cytb* marker, GenBank Accession Numbers for sequences generated here or downloaded from GenBank are also provided. The respective published literature for those sequences that were downloaded from GenBank (†) is given in the footnote below the table.

Code		Subspecies		Locality	Country	ddRAD	cytb
	Heckes et al. 2005	Golay et al. 1993	Tomović, 2006				
This study							
8	meridionalis	meridionalis	Transition zone	Aspropotamos river, Trikala	Greece		OP973940
32	meridionalis	meridionalis	montandoni	Kerkini, Serres	Greece	YES	OP973941
33	meridionalis	meridionalis	montandoni	Stavrochori, Kilkis	Greece	YES	OP973942
34	meridionalis	meridionalis	Undetermined	Kerkyra island	Greece		OP973943
35	meridionalis	meridionalis	Transition zone	Monastery Spilia, Karditsa	Greece		OP973944
80	meridionalis	meridionalis	meridionalis	Geraki, Peloponnesos	Greece	YES	OP973945
94	meridionalis	meridionalis	meridionalis	Dyrrachi, Peloponnesos	Greece		OP973946
99	meridionalis	meridionalis	meridionalis	Dorio, Peloponnesos	Greece	YES	OP973947
102	meridionalis	meridionalis	meridionalis	Kyparisia, Peloponnesos	Greece	YES	OP973948
363	meridionalis	meridionalis	meridionalis	Erymanthos Mt, Peloponnesos	Greece	YES	OP973949
414	meridionalis	meridionalis	meridionalis	Tinos island	Greece	YES	OP973950
417	meridionalis	meridionalis	meridionalis	Tinos island	Greece	YES	OP973951
423	meridionalis	meridionalis	meridionalis	Souli, Peloponnesos	Greece	YES	OP973952
428	meridionalis	meridionalis	meridionalis	Louros, Mesologhi	Greece	YES	OP973953
452	meridionalis	meridionalis	meridionalis	Antiparos island	Greece	YES	OP973954
465	meridionalis	meridionalis	meridionalis	Thermo	Greece	YES	OP973955
472	meridionalis	meridionalis	Transition zone	Morfi, Parga	Greece	YES	OP973956
474	meridionalis	meridionalis	Transition zone	Livadari, Parga	Greece	YES	OP973957
494	meridionalis	meridionalis	meridionalis	Dystou Lake, Evvoia	Greece	YES	OP973958
499	meridionalis	meridionalis	Transition zone	Metaxochori	Greece	YES	OP973959
507	ammodytes	ammodytes	ammodytes	Mandre, Pag	Croatia	YES	OP973960
509	meridionalis	meridionalis	meridionalis	Syros island	Greece	YES	OP973961
512	meridionalis	meridionalis	meridionalis	Syros island	Greece	YES	OP973962
526	meridionalis	meridionalis	meridionalis	Antiparos island	Greece	YES	OP973963
529	meridionalis	meridionalis	meridionalis	Naxos island	Greece	YES	OP973964
543	meridionalis	meridionalis	meridionalis	Koumarias, Peloponnesos	Greece	YES	OP973965
546	meridionalis	meridionalis	meridionalis	Neochórion, , Peloponnesos	Greece	YES	OP973966

551   meridionalis   meridionalis   Argyo, Evoia   Greece   YES   OP973963     552   meridionalis   meridionalis   Choni, Evoia   Greece   YES   OP973966     562   meridionalis   meridionalis   Sofko, Peoponesos   Greece   YES   OP973970     568   meridionalis   meridionalis   Sofko, Peoponesos   Greece   YES   OP973971     575   meridionalis   meridionalis   Transition zone   Karla Lake   Greece   YES   OP973972     592   meridionalis   meridionalis   Transition zone   Karla Lake   Greece   YES   OP973973     593   meridionalis   meridionalis   meridionalis   Planitcro, Pelponnesos   Greece   YES   OP973973     631   anmodytes   anmodytes   anmodytes   Male Paklenica   Croatia   OP973975     632   anmodytes   anmodytes   monodytes   Greece   YES   OP973976     763   anmodytes   anmodytes   anmodytes   Greece   YES   OP973976     764   anmodytes   anmodytes   anmodytes   Greece   YES   OP973978     793   montandoni   montandoni   Revo   Greece								
555   meridionalis   meridionalis   meridionalis   Coni, Evvoia   Greece   YES   OP973968     562   meridionalis   meridionalis   Zanks, Evvoia   Greece   YES   OP973970     568   meridionalis   meridionalis   meridionalis   Sofko, Peloponesos   Greece   YES   OP973971     575   meridionalis   meridionalis   Transition zone   Anygdali, Ossa   Greece   YES   OP973973     592   meridionalis   meridionalis   Transition zone   Kasti, Ossa   Greece   YES   OP973971     593   meridionalis   meridionalis   meridionalis   Kasti, Ossa   Greece   YES   OP973973     594   meridionalis   meridionalis   meridionalis   Kasti, Ossa   Greece   YES   OP973973     503   ammodytes   ammodytes   manuotytes   Mala Paklenica, Ramiči   Croatia   OP973971     575   ammodytes   ammodytes   ammodytes   Eibenthal   Romania   YES   OP973979     751   ammodytes   ammodytes   ammodytes   motandoni   Par03973   OP973981     793   motandoni   motandoni   motandoni   Recovo   Bulgaria   OP973981 <	551	meridionalis	meridionalis	meridionalis	Argyro, Evvoia	Greece	YES	OP973967
562   meridionalis   meridionalis   meridionalis   Sofiko, Peloponnesos   Greece   YES   OP973909     568   meridionalis   meridionalis   Sofiko, Peloponnesos   Greece   YES   OP973971     570   meridionalis   meridionalis   Transition zone   Anygali, Osa   Greece   YES   OP973972     592   meridionalis   meridionalis   Transition zone   Kastri, Osa   Greece   YES   OP973973     594   meridionalis   meridionalis   Ranidianis   Ristri, Osa   Greece   YES   OP973976     593   meridionalis   meridionalis   meridionalis   Kastri, Lake   Greece   YES   OP973976     631   anmodytes   anmodytes   mondytes   Mula Paklenica   Croatia   OP973976     632   anmodytes   anmodytes   anmodytes   mondytes   Electual   Romania   YES   OP973978     753   montadoni   meridionalis   montandoni   Rescov   Bulgaria   OP97398     793   montandoni   motandoni   Rotanai   YES   OP973981     794   meridionalis   motandoni   Rotanai   YES   OP973981     7953   motandoni   mo	555	meridionalis	meridionalis	meridionalis	Choni, Evvoia	Greece	YES	OP973968
568     meridionalis     meridionalis     meridionalis     Avlona, Attiki     Greece     YES     OP973970       559     meridionalis     meridionalis     meridionalis     Transition zone     Avlona, Attiki     Greece     YES     OP973971       551     meridionalis     meridionalis     Transition zone     Kastri, Ossa     Greece     YES     OP973973       594     meridionalis     meridionalis     meridionalis     Meridionalis     Plantero, Ploponnesos     Greece     YES     OP973976       501     ammodytes     ammodytes     ammodytes     Plantero, Ploponnesos     Greece     YES     OP973976       512     ammodytes     ammodytes     ammodytes     Plantero, Ploponnesos     Greece     YES     OP973977       561     ammodytes     ammodytes     ammodytes     Elbenthal     Romania     YES     OP973971       753     ammodytes     ammodytes     ammodytes     montandoni     Pottors     Greece     YES     OP973981       754     ammodytes     ammodytes     montandoni     motandoni     Rotanadoni     Avlona     OP973982       793	562	meridionalis	meridionalis	meridionalis	Zarakes, Evvoia	Greece	YES	OP973969
569   meridionalis   meridionalis   meridionalis   meridionalis   Transition zone   Avyalai, Ossa   Greece   YES   OP973971     575   meridionalis   meridionalis   Transition zone   Kastri, Ossa   Greece   YES   OP973971     594   meridionalis   meridionalis   Transition zone   Kastri, Ossa   Greece   YES   OP973973     591   meridionalis   meridionalis   meridionalis   Plateinea, Ramići   Croatia   OP973971     631   anmodytes   anmodytes   anmodytes   anmodytes   OP973971     753   anmodytes   anmodytes   anmodytes   Eibenthal   Romania   YES   OP973973     754   montandoni   meridionalis   montandoni   Recovo   Bulgaria   OP973981     793   montandoni   montandoni   montandoni   Recovo   Bulgaria   OP973983     794   meridionalis or   meridionalis or   montandoni   Poprase   OP973983     795   meridionalis or   montandoni   Poprase   OP973983     796   meridionalis or   montandoni   Recovo   Bulgaria   OP973983     797   meridionalis or   montandoni   Pontandoni   Po	568	meridionalis	meridionalis	meridionalis	Sofiko, Peloponnesos	Greece	YES	OP973970
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592   meridionalis   meridionalis   Transition zone   Kastri, Ossa   Greece   YES   OP973973     594   meridionalis   meridionalis   meridionalis   meridionalis   Greece   YES   OP973976     597   meridionalis   meridionalis   meridionalis   Planitero, Peloponesos   Greece   YES   OP973977     631   anmodytes   anmodytes   anmodytes   Planitero, Peloponesos   Greece   YES   OP973977     632   anmodytes   anmodytes   Eibenthal   Romania   YES   OP973977     753   anmodytes   anmodytes   Eibenthal   Romania   OP9739797     761   montandoni   montandoni   montandoni   Review   Bulgaria   OP973978     793   montandoni   montandoni   Reviewo   Bulgaria   OP973981     794   montandoni   montandoni   Reviewo   Bulgaria   OP973984     795   meridionalis   montandoni   Reviewo   Bulgaria   OP973984     794   meridionalis   montandoni   Reviewo   Bulgaria   OP973984     795   meridionalis   montandoni   Spitalitik   OP973984     798   meridionalis   meridi	575	meridionalis	meridionalis	Transition zone	Amygdali, Ossa	Greece	YES	OP973972
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757anmodytesanmodytesEicenthalRomaniaOP97397761montandonimeridionalismontandoniLoutrosGreeceYESOP973981793montandonimontandoniRezovoBulgariaOP973981799montandonimontandoniRezovoBulgariaOP973982899meridionalis ormeridionalis ormontandoniStalterAlbaniaYESOP9739831466meridionalis ormeridionalis ormontandoniProhorSerbiaOP9739871516meridionalismeridionalismontandoniAsmata, Prespansko jezeroNorth MacedoniaYESOP9739871521meridionalismeridionalismontandoniAsmata, Prespansko jezeroNorth MacedoniaYESOP9739871534meridionalismeridionalismontandoniAsmata, Prespansko jezeroNorth MacedoniaYESOP973987154meridionalismeridionalismeridionalismeridionalisYESOP9739871554meridionalismeridionalismeridionalisYESOP9739871584anmodytes orTansition zoneRadikaNorth MacedoniaYESOP9739911998meridionalismeridionalismeridionalisExochori, PeloponnesosGreeceYESOP9739922277meridionalismeridionalismeridionalismeridionalisYESOP9739912276meridionalismeridionalismeridionalismeridionalis <t< td=""><td>756</td><td>ammodytes</td><td>ammodytes</td><td>ammodytes</td><td>Eibenthal</td><td>Romania</td><td>YES</td><td>OP973978</td></t<>	756	ammodytes	ammodytes	ammodytes	Eibenthal	Romania	YES	OP973978
761montandonimontandonimontandoniLottosGreeceYESOP973980793montandonimontandonimontandoniRezovoBulgariaOP973981799montandonimontandoniRezovoBulgariaOP973982880meridionalismeridionalismontandoniSyri i KaltërAlbaniaYESOP973982980meridionalismeridionalismontandoniSyri i KaltërAlbaniaYESOP9739831460meridionalismeridionalismontandoniNichichNorth MacedoniaYESOP9739861511meridionalismeridionalismontandoniGipocheliNorth MacedoniaYESOP9739861629meridionalismeridionalismontandoniAsamati, Prespansko jezeroNorth MacedoniaYESOP9739871754meridionalismeridionalisTransition zoneRaikaNorth MacedoniaYESOP9739801958anmodytesanmodytes orTransition zoneRaikaNorth MacedoniaYESOP9739912276meridionalismeridionalismeridionalisExochori, PeloponnesosGreeceYESOP9739922277meridionalismeridionalismeridionalisExochori, PeloponnesosGreeceYESOP9739922455ammodytes orramsition zoneVitenNorth MacedoniaYESOP9739922457ammodytesammodytesammodytesammodytesOP9739962577 <td< td=""><td>757</td><td>ammodytes</td><td>ammodytes</td><td>ammodytes</td><td>Eibenthal</td><td>Romania</td><td></td><td>OP973979</td></td<>	757	ammodytes	ammodytes	ammodytes	Eibenthal	Romania		OP973979
793montandonimontandonimontandoniRezovoBulgariaOP973981799montandonimontandoniRezovoBulgariaOP973982798meridionalismontandoniSyri i KaltërAlbaniaYESOP9739831466meridionalis ormeridionalis ormontandoniSyri i KaltërAlbaniaYESOP9739841466meridionalis ormeridionalismontandoniProhorSerbiaOP9739841516meridionalismeridionalismontandoniGipopheliNorth MacedoniaYESOP9739861571meridionalismeridionalismontandoniGipopheliNorth MacedoniaYESOP9739861629meridionalismeridionalismontandoniGipopheliNorth MacedoniaYESOP9739871754meridionalismeridionalisTransition zoneRibnicaNorth MacedoniaYESOP9739891958ammodytes orammodytes orTransition zoneRadikaNorth MacedoniaYESOP9739902276meridionalismeridionalismeridionalisExochori, PeloponnesosGreeceYESOP9739912277meridionalismeridionalismeridionalisExochori, PeloponnesosGreeceYESOP9739932455ammodytes orammodytesammodytesammodytesOP973994S2847ammodytesammodytesammodytesAmmodytesOP9739952848ammodytesammodytes <td< td=""><td>761</td><td>montandoni</td><td>meridionalis</td><td>montandoni</td><td>Loutros</td><td>Greece</td><td>YES</td><td>OP973980</td></td<>	761	montandoni	meridionalis	montandoni	Loutros	Greece	YES	OP973980
799montandonimontandoniRezvoBulgariaOP973982989meridionalismeridionalismontandoniSyri KaltërAlbaniaYESOP9739831466meridionalis ormeridionalis ormontandoniProhorSerbiaOP9739841516meridionalismeridionalismontandoniGjopcheliNorth MacedoniaYESOP9739861571meridionalismeridionalismontandoniGjopcheliNorth MacedoniaYESOP9739861629meridionalismeridionalismontandoniAsamati, Prespansko jezeroNorth MacedoniaYESOP9739871754meridionalismeridionalisTransition zoneRibnicaNorth MacedoniaYESOP9739871958ammodytesammodytesammodytesVitoja, Skadar LakeMontenegroYESOP9739891998ammodytes orammodytes orTransition zoneRadikaNorth MacedoniaYESOP9739912276meridionalismeridionalismeridionalisExochori, PeloponnesosGreeceYESOP9739922277meridionalismeridionalismeridionalisExochori, PeloponnesosGreeceYESOP9739932455ammodytes orammodytes orTransition zoneVrbenOP973995OP9739952457ammodytesammodytesammodytesBokumirskoMontenegroYESOP9739952457ammodytesammodytesammodytesammodytesOP973995 <td>793</td> <td>montandoni</td> <td>montandoni</td> <td>montandoni</td> <td>Rezovo</td> <td>Bulgaria</td> <td></td> <td>OP973981</td>	793	montandoni	montandoni	montandoni	Rezovo	Bulgaria		OP973981
989meridionalis meridionalis or meridionalis or meridionalis or meridionalis or meridionalis or meridionalis or meridionalis or meridionalis or meridionalis or meridionalismontandoni montandoniSyri i Kaltër ProhorAlbaniaYES SerbiaOP973983 OP973983 OP9739841516meridionalis meridionalismeridionalis meridionalismontandoniNikolichNorth MacedoniaYES OP973986OP9739851571meridionalis meridionalismeridionalis meridionalismontandoniAsamati, Prespansko jezero North MacedoniaYES OP973987OP9739871754meridionalis meridionalismeridionalis meridionalisTransition zoneRibnicaNorth MacedoniaYES OP9739881958anmodytes or meridionalismeridionalis meridionalisTransition zoneRadika Exochori, PeloponnesosGreeceYES OP9739912276meridionalis meridionalismeridionalis meridionalismeridionalis meridionalisCP9739922455 anmodytes or meridionalismeridionalis meridionalisExochori, Peloponnesos report reportGreeceYES OP9739922457anmodytes anmodytesanmodytes anmodytesanmodytes anmodytesMontenegroYES OP9739932484anmodytes anmodytesanmodytes anmodytesanmodytes anmodytesMontenegroYES OP973993257.1meridionalis meridionalisanmodytes anmodytesMontenegroYES OP973993253.2anmodytes anmodyte	799	montandoni	montandoni	montandoni	Rezovo	Bulgaria		OP973982
1466meridionalis or anmodytesmontandoni anmodytesProhorSerbiaOP9739841516meridionalismontdytesmontandoniNikolichNorth MacedoniaYESOP9739851571meridionalismeridionalismontandoniGjopcheliNorth MacedoniaYESOP9739861629meridionalismeridionalismontandoniAsamati, Prespansko jezeroNorth MacedoniaYESOP9739871754meridionalismeridionalismontandoniAsamati, Prespansko jezeroNorth MacedoniaYESOP9739871754meridionalismeridionalisTransition zoneRibnicaNorth MacedoniaYESOP9739871958anmodytes oranmodytes orTransition zoneRadikaNorth MacedoniaYESOP9739912276meridionalismeridionalismeridionalisExochori, PeloponnesosGreeceYESOP9739922277meridionalismeridionalismeridionalisExochori, PeloponnesosGreeceYESOP9739922277meridionalismeridionalismeridionalisExochori, PeloponnesosGreeceYESOP9739932455anmodytesanmodytesanmodytesanmodytesOp973996Op9739953157meridionalismontadoniOrikumAlbaniaYESOP9739953157anmodytesanmodytesanmodytesanmodytesOp9739963157meridionalismontadoniOrikumAlbaniaYES	989	meridionalis	meridionalis	montandoni	Syri i Kaltër	Albania	YES	OP973983
animodytesanimodytesanimodytesveridionalismontandoniNikolichNorth MacedoniaYESOP9739851511meridionalismeridionalismontandoniGjopcheliNorth MacedoniaYESOP9739871629meridionalismeridionalismontandoniAsamati, Prespansko jezeroNorth MacedoniaYESOP9739871754meridionalismeridionalisTransition zoneRibnicaNorth MacedoniaYESOP9739891754meridionalismeridionalisTransition zoneRibnicaNorth MacedoniaYESOP9739891958anmodytesanmodytesTransition zoneRadikaNorth MacedoniaYESOP9739891958anmodytesanmodytesTransition zoneRadikaNorth MacedoniaYESOP9739912276meridionalismeridionalismeridionalisExochori, PeloponnesosGreeceYESOP9739912277meridionalismeridionalismeridionalisExochori, PeloponnesosGreeceYESOP9739942475anmodytes oranmodytes orTransition zoneVtbenNorth MacedoniaYESOP9739942848anmodytesanmodytesanmodytesBokumirskoMontenegroYESOP9739943157anmodytesanmodytesanmodytesBokumirskoMontenegroYESOP9739973757meridionalismeridionalismontandoniOrikumAlbaniaYESOP9739973757 <t< td=""><td>1466</td><td>meridionalis or</td><td>meridionalis or</td><td>montandoni</td><td>Prohor</td><td>Serbia</td><td></td><td>OP973984</td></t<>	1466	meridionalis or	meridionalis or	montandoni	Prohor	Serbia		OP973984
1516meridionalismeridionalismontandoniNikolichNorth MacedoniaYESOP9739851571meridionalismeridionalismontandoniGjopcheliNorth MacedoniaYESOP9739871629meridionalismeridionalismontandoniAsamati, Prespansko jezeroNorth MacedoniaYESOP9739871754meridionalismeridionalisTransition zoneRibnicaNorth MacedoniaYESOP9739891958ammodytesammodytesammodytesVitoja, Skadar LakeMontenegroYESOP9739891998ammodytes orTransition zoneRadikaNorth MacedoniaYESOP9739912276meridionalismeridionalismeridionalisExochori, PeloponnesosGreeceYESOP9739922277meridionalismeridionalismeridionalisExochori, PeloponnesosGreeceYESOP9739922455ammodytesammodytesmandytesKreeceYESOP9739942847ammodytesammodytesammodytesMontenegroYESOP9739953157ammodytesammodytesammodytesBokumirskoMontenegroYESOP9739963157meridionalismeridionalismontandoniOrikumAlbaniaYESOP9739963157ammodytesammodytesammodytesCetinjeMontenegroYESOP9739963157meridionalismeridionalismontandoniOrikumAlbaniaYESOP		ammodytes	ammodytes					
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1629meridionalismeridionalismontandoniAsamati, Prespansko jezeroNorth MacedoniaYESOP9739871754meridionalismeridionalisTransition zoneRibnicaNorth MacedoniaYESOP9739881958ammodytesammodytes orammodytesWioja, Skadar LakeMontenegroYESOP9739891998ammodytes orammodytes orTransition zoneRadikaNorth MacedoniaYESOP9739802276meridionalismeridionalismeridionalismeridionalisSop973991Sop9739912277meridionalismeridionalismeridionalisExochori, PeloponnesosGreeceYESOP9739922475ammodytes orammodytes orTransition zoneVrbenOP973993OP9739932484ammodytesammodytesammodytesBokumirskoMontenegroYESOP9739942847ammodytesammodytesammodytesBokumirskoMontenegroYESOP9739953157ammodytesammodytesammodytesDucaj, ProkletijeAlbaniaYESOP9739963757meridionalismeridionalismondytesammodytesCetinjeMontenegroYESOP9739984455ammodytesammodytesammodytesGreityAlbaniaYESOP9739963757meridionalismeridionalismondytesammodytesZetinjeMontenegroYESOP9739984455ammodytesammodytesammodytes<	1571	meridionalis	meridionalis	montandoni	Gjopcheli	North Macedonia		OP973986
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1958ammodytesammodytesammodytesammodytesammodytesorYESOP9739891998ammodytes orammodytes orTransition zoneRadikaNorth MacedoniaYESOP9739902276meridionalismeridionalismeridionalismeridionalisExochori, PeloponnesosGreeceYESOP9739922277meridionalismeridionalismeridionalisExochori, PeloponnesosGreeceYESOP9739922455anmodytes orammodytes orTransition zoneVrbenNorth MacedoniaOP9739932457ammodytesammodytesTransition zoneVrbenNorth MacedoniaOP9739942848ammodytesammodytesammodytesBokumirskoMontenegroYESOP9739952847ammodytesammodytesammodytesBokumirskoMontenegroYESOP9739942848ammodytesammodytesammodytesBokumirskoMontenegroYESOP9739953157ammodytesammodytesammodytesBucaj, ProkletijeAlbaniaYESOP9739974455ammodytesammodytesammodytesammodytesCetinjeMontenegroYESOP9739984481ammodytesammodytesammodytesammodytesCetinjeMontenegroYESOP9739994481ammodytesammodytesammodytesammodytesCetinjeMontenegroYESOP9739994481ammodytesammodytesam	1754	meridionalis	meridionalis	Transition zone	Ribnica	North Macedonia	YES	OP973988
1998ammodytes or meridionalisammodytes or meridionalisTransition zone meridionalisRadikaNorth MacedoniaYESOP9739902276meridionalismeridionalismeridionalisExochori, PeloponnesosGreeceYESOP9739912277meridionalismeridionalismeridionalisExochori, PeloponnesosGreeceYESOP9739922277meridionalismeridionalismeridionalisExochori, PeloponnesosGreeceYESOP9739932455ammodytes or meridionalisammodytes or meridionalisTransition zoneVrbenNorth MacedoniaOP9739942847ammodytesammodytesammodytesammodytesBokumirskoMontenegroYESOP9739942848ammodytesammodytesammodytesammodytesBokumirskoMontenegroYESOP9739963157ammodytesammodytesammodytesDucaj, ProkletijeAlbaniaYESOP9739973157ammodytesammodytesammodytesDucaj, ProkletijeAlbaniaYESOP9739974455ammodytesammodytesammodytesZanjev DoMontenegroYESOP9739944481ammodytesammodytesammodytesAmmodytesZanjev DoMontenegroYESOP9739994814ammodytesammodytesammodytesAmmodytesZanjev DoMontenegroYESOP9740004932meridionalismeridionalismotandoniBilisht <td< td=""><td>1958</td><td>ammodytes</td><td>ammodytes</td><td>ammodytes</td><td>Vitoja, Skadar Lake</td><td>Montenegro</td><td>YES</td><td>OP973989</td></td<>	1958	ammodytes	ammodytes	ammodytes	Vitoja, Skadar Lake	Montenegro	YES	OP973989
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2276meridionalismeridionalismeridionalisExochori, PeloponnesosGreeceYESOP9739912277meridionalismeridionalismeridionalisExochori, PeloponnesosGreeceYESOP9739922455ammodytes or meridionalismeridionalisTransition zoneVrbenNorth MacedoniaOP9739932847ammodytesammodytesammodytesammodytesBokumirskoMontenegroYESOP9739942848ammodytesammodytesammodytesBokumirskoMontenegroYESOP9739953157ammodytesammodytesammodytesBokumirskoMontenegroYESOP9739963757meridionalismeridionalismontandoniOrikumAlbaniaYESOP9739974455ammodytesammodytesCetinjeMontenegroYESOP9739984481ammodytesammodytesammodytesZanjev DoMontenegroYESOP9739974451ammodytesammodytesammodytesZanjev DoMontenegroYESOP9739984481ammodytesammodytesammodytesZanjev DoMontenegroYESOP9739974452meridionalismeridionalismontandoniBilishtAlbaniaYESOP9740004932meridionalismeridionalismontandoniBilishtAlbaniaYESOP9740015681meridionalismeridionalismeridionalismeridionalisLimeniGreece<		meridionalis	meridionalis					
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2455ammodytes or meridionalisammodytes or meridionalisTransition zoneVrbenNorth MacedoniaOP9739932847ammodytesammodytesammodytesBokumirskoMontenegroYESOP9739942848ammodytesammodytesammodytesBokumirskoMontenegroYESOP9739953157ammodytesammodytesammodytesDucaj, ProkletijeAlbaniaYESOP9739963757meridionalismeridionalismontandoniOrikumAlbaniaYESOP9739974455ammodytesammodytesammodytesCetinjeMontenegroYESOP9739984481ammodytesammodytesammodytesZanjev DoMontenegroYESOP9739994814ammodytesammodytesammodytesGryke LugjeAlbaniaYESOP9740004932meridionalismeridionalismontandoniBilishtAlbaniaYESOP9740015681meridionalismeridionalismontandoniVIlahineAlbaniaOP9740025800meridionalismeridionalismeridionalisLimeniGreeceYESOP974003	2277	meridionalis	meridionalis	meridionalis	Exochori, Peloponnesos	Greece	YES	OP973992
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4481ammodytesammodytesammodytesŽanjev DoMontenegroYESOP9739994814ammodytesammodytesammodytesGryke LugjeAlbaniaYESOP9740004932meridionalismeridionalismontandoniBilishtAlbaniaYESOP9740015681meridionalismeridionalismontandoniVllahineAlbaniaOP9740025800meridionalismeridionalismeridionalisLimeniGreeceYESOP974003	4455	ammodytes	ammodytes	ammodytes	Cetinje	Montenegro	YES	OP973998
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5800 <i>meridionalis meridionalis meridionalis</i> Limeni Greece YES OP974003	5681	meridionalis	meridionalis	montandoni	Vllahine	Albania		OP974002
	5800	meridionalis	meridionalis	meridionalis	Limeni	Greece	YES	OP974003

6308	montandoni	meridionalis	montandoni	Lozenets	Bulgaria	YES	OP974004
6479	ammodytes or	ammodytes or	Transition zone	NP Dajti	Albania	YES	OP974005
	meridionalis	meridionalis		3			
7030	ammodytes	ammodytes	ammodytes	Ckla	Montenegro	YES	OP974006
7096	meridionalis	meridionalis	montandoni	Near Kutal	Albania	YES	OP974007
7139	ammodytes	ammodytes	ammodytes	Krk	Croatia		OP974008
7567	ammodytes	ammodytes	ammodytes	between Draglica - Kokin	Serbia	YES	OP974009
7568	ammodytes	ammodytes	ammodytes	between Draglica - Kokin	Serbia		OP974010
7569	ammodytes	ammodytes	ammodytes	between Draglica - Kokin	Serbia	YES	OP974011
8415	ammodytes	ammodytes	ammodytes	Soča	Slovenia		OP974012
JIR127	transcaucasiana	transcaucasiana	transcaucasiana	Borjomi-Kharagauli	Georgia	YES	OP974013
<b>Previously Publis</b>	shed			× ×	*		
KR153575	ammodytes	ammodvtes	ammodytes	Nadiza Valley	Slovenia		KR153575
KR153576	ammodytes	ammodytes	ammodytes	Nadiza Valley	Slovenia		KR153576
DO186477	transcaucasiana or	transcaucasiana	transcaucasiana	Village Zara east of Sivas	Turkey		DO186477
	montandoni	or meridionalis	or montandoni	C	2		
DQ186478	transcaucasiana	transcaucasiana	transcaucasiana	Kura valley (Noyemberyan)	Armenia		DQ186478
DQ186479	transcaucasiana	transcaucasiana	transcaucasiana	Aralik	Turkey		DQ186479
DQ186480	meridionalis	meridionalis	Transition zone	Damasi, Olympos	Greece		DQ186480
DQ186481	meridionalis	meridionalis	Transition zone	Ossa	Greece		DQ186481
DQ186482	meridionalis	meridionalis	montandoni	Chalkidiki	Greece		DQ186482
DQ186482	meridionalis	meridionalis	Transition zone	Olympos	Greece		DQ186482
DQ186482	meridionalis	meridionalis	meridionalis	Nafpaktos	Greece		DQ186482
DQ186483	meridionalis	meridionalis	montandoni	Crnovska River, Trgoviste	Serbia		DQ186483
DQ186484	meridionalis	meridionalis	montandoni	Nov Dojran	North Macedonia		DQ186484
DQ186485	meridionalis	meridionalis	montandoni	Prespa lake	Greece		DQ186485
DQ186486	meridionalis	meridionalis	meridionalis	Lamia	Greece		DQ186486
DQ186487	meridionalis	meridionalis	meridionalis	North Evvoia	Greece		DQ186487
DQ186488	montandoni	meridionalis	montandoni	North costal area	Bulgaria		DQ186488
DQ186488	montandoni	meridionalis	montandoni	Tsarevo	Bulgaria		DQ186488
DQ186489	montandoni	meridionalis	montandoni	Ahtopol	Bulgaria		DQ186489
DQ186490	montandoni	meridionalis	montandoni	Village Sadovec, Plevensko	Bulgaria		DQ186490
DQ186491	montandoni	meridionalis	montandoni	Village Nadezden, Harmanlijsko	Bulgaria		DQ186491
DQ186492	montandoni	meridionalis	montandoni	Sapanca, Asia Minor	Turkey		DQ186492
DQ186493	meridionalis	meridionalis	meridionalis	Kardamili	Greece		DQ186493
DQ186494	meridionalis	meridionalis	meridionalis	Feneos Bazin	Greece		DQ186494
DQ186495	meridionalis	meridionalis	meridionalis	Kyparissia	Greece		DQ186495
DQ186496	meridionalis	meridionalis	meridionalis	Syros island, Cyclades	Greece		DQ186496
DQ186497	meridionalis	meridionalis	meridionalis	Tinos island, Cyclades	Greece		DQ186497

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AY311380 montandoni meridionalis montandoni Unkwown Bulgaria AY3	/311380
AY311381 ammodytes ammodytes ammodytes Unkwown Former Yugoslavia AY3	/311381
AY311382 montandoni meridionalis montandoni Unkwown Bulgaria AY3	/311382

EU624314	ammodytes	ammodytes	ammodytes	Unkwown	Former Yugoslavia		EU624314
DQ897771	ammodytes	ammodytes	ammodytes	Unkwown	Former Yugoslavia		DQ897771
Outgroup							
3162	Vipera berus					YES	OP974014
4187	Vipera berus					YES	OP974015
NC036956	Vipera berus						NC036956
KX345256	Vipera berus						KX345256
DQ186476	Vipera berus						DQ186476
AY611997	Vipera aspis						AY611997
KR153577	Vipera aspis						KR153577

†Sequences of the following GenBank Accession Numbers have been originally published here:

DQ186477-DQ186520 (Ursenbacher et al., 2008), AY311379-AY311382 (Garrigues et al., 2005), EU624314 (Wüster et al., 2008), DQ897771 (Wüster et al., 2007), NC036956 (Gao et al., 2018), KX345256 (Cui et al., 2016), AY611997 (Nagy et al., 2005), KR153575 & KR153577 (Mebert et al., 2015).

**Table S2.** Parameters used during de-multiplexing and filtering with iPyRAD and a summary of all resulted datasets used for the analyses performed in this study. Samples were demultiplexed using their unique Illumina barcode and adaptor sequences, and each read was reduced to a maximum of 39 bp after removing the 6 bp restriction site overhang and the 5 bp barcode. Sites with Phred quality scores under 99% (Phred score = 20) were changed into "N" characters, and reads with <10% N's were discarded. The filtered reads for each sample were clustered using VSEARCH 2.4.3 (Rognes et al., 2016) and aligned with MUSCLE 3.8.31 (Edgar, 2004). We assembled the ddRADseq data using a clustering threshold of 92%, in order to reduce the risk of combining paralogs, while still accommodating a realistic level of sequence variation (Leaché & Rannala, 2011). As additional filtering steps, consensus sequences with low coverage (< 6 reads), excessive undetermined or heterozygous sites (> 4), excessive indels (> 1) or too many haplotypes (> 2) were discarded. The consensus sequences were clustered across samples using the same clustering threshold (92%). Again, alignment was done with MUSCLE, applying a paralog filter that removes loci with excessive shared heterozygosity among samples (paralog filter = 200). To minimize the possibility of returning paralogs, the maximum number of single nucleotide polymorphisms (SNPs) per locus was set to 15 (default value 20). For final datasets we used two levels of missing data, expressed as the minimum number of individuals with data for a given locus: 100% (each locus present in all samples) and 75% (each locus present in at least 75% of the samples). The specific dataset used in each analysis is highlighted in gray. The number of *V. berus* outgroup samples that were included in each dataset is given in parentheses. The last column includes the percentage of missing data per sample in the final datasets.

Parameter	Samples	Prefiltered	Filtered	Invariable	Max SNPs	Base	SNPs	uSNPs	Biallelic	Missing data
Dataset	(V. berus)	loci	loci	loci	per locus	pairs			uSNPs	per sample
STRUCTURE-All-100	57 (0)	48,437	976	302	6	32,250	1,294	674	-	0
STRUCTURE-North-100	15 (0)	26,844	2,446	1,456	5	80,718	1,417	990	-	0
STRUCTURE-South-100	42 (0)	37,744	1,730	730	10	57,127	1,726	1,000	-	0
SNAPP-100	20 (2)	35,707	2,885	1,004	7	95,232	3,433	1,881	1,847	0
SNAPP-75	20 (2)	35,707	7,373	2,604	10	243,527	9,096	4,769	2,929	1%-19%
SVDquartets-100	44 (2)	45,831	1,156	325	7	38,164	1,679	831	-	0
SVDquartets-75	44 (2)	45,831	6,285	1,749	10	207,849	9,593	4,536	-	1%-38%
ML-Concatenation-75	44 (2)	45,831	6,285	1,749	10	207,849	9,593	4,536	-	1%-38%
TREEMIX-75	57 (0)	48,437	5,991	1,794	10	198,045	8,663	4,197	-	1%-41%

**Table S3.** Results of the four-population (*f4*) tests, 630 in total, performed among populations of *Vipera ammodytes* that indicated deviation from "treeness" and possible introgression. The test shows whether a four-branched simple tree of the form (A, B; C, D), depicting relationships among four selected populations (pop1 to pop4), may be rejected based on the true phylogenetic relationships estimated with TREEMIX. For each test, we give the value of the *f4* statistics, the respective standard deviation (sd) and the deviation of *f4* from zero in units of the standard error (z). The respective four-branched tree is rejected when it returns high ( $\geq$ 3 or  $\leq$ -3), non-zero values of z.

pop1	pop2	pop3	pop4	f4	sd	Z
Caucasus	East	Peloponnesos	South 2	0.003499	0.000831	4.21297
Caucasus	East	South 1	South 2	0.002346	0.000576	4.07594
Caucasus	East	South 2	Southwest	-0.00299	0.000642	-4.65891

**Table S4.** Probability records of five alternative topologies (rows) tested with IQ-TREE, using seven different methodologies, presented in the footnotes and the last seven columns. LogL gives the likelihood value estimated for the test-tree, i.e. a tree constrained to represent a specific hypothesis, and deltaL shows the difference between this value and the maximal logL in the set, i.e. the unconstrained topology. Plus signs (+) denote the 95% confidence sets. Minus signs (-) denote significant exclusion. The alternative topology was rejected when all seven methodologies returned a minus sign.

Topology Test	logL	deltaL	bp-RELL	p-KH	p-SH	p-WKH	p-WSH	c-ELW	p-AU
"NORTH"monophyletic	-3155.862	1.323	0.266 +	0.255 +	0.919 +	0.265 +	0.802 +	0.284 +	0.374 +
"East + Caucasus" monophyletic	-3161.687	3.649	0.087 +	0.126 +	0.836 +	0.126 +	0.436 +	0.106 +	0.123 +
"South-1" and "South-2" each monophyletic	-3159.259	1.221	0.305 +	0.275 +	0.933 +	0.275 +	0.835 +	0.309 +	0.327 +
"South (1+2) + Cyclades" sister clades	-3222.858	64.822	0 -	0.0001 -	0.0001 -	0 -	0 -	8.86e-14 -	0.00021 -
"South 1+ Cyclades" sister clades	-3222.860	64.822	0 -	0.0001 -	0.0001 -	0 -	0 -	8.83e-14 -	0.00022 -

bp-RELL: bootstrap proportion using RELL method (Kishino et al., 1990).

p-KH: p-value of one sided KH test (Kishino & Hasegawa, 1989).

p-SH: p-value of SH test (Shimodaira & Hasegawa, 1999).

p-WKH: p-value of weighted KH test.

p-WSH: p-value of weighted SH test.

c-ELW: Expected Likelihood Weight (Strimmer & Rambaut, 2002).

p-AU: p-value of approximately unbiased (AU) test (Shimodaira, 2002).

**Table S5.** Relative (%) importance of each explanatory variable of present climatic and topographical conditions used in our final Species Distribution Models (SDMs) with MaxEnt (Phillips et al., 2006) – through the R package "dismo" v.1.1.4 (Hijmans et al., 2011) – to predict habitat suitability for *Vipera ammodytes* based on georeferenced presence-points in total (ALL) and divided in five groups: Cyclades Islands, Asia, South, North, and South grouped with Asia. We applied a thinning process of 10 replicates in order to randomly keep one occurrence within a radius of 5km for the minimization of the effects of sampling bias, using the "spThin" R package (Aiello-Lammens et al., 2015). The respective number of presence records used to build models is given before and after the thinning process. Preliminary models were run for each group in order to (1) account for correlation in variables by setting a limit of correlation (Pearson's correlation coefficient  $r^2 > 0.75$ ) and keep only one from each group of auto-correlated variables, (2) avoid model overfitting by fine-tuning the regularization multiplier (RM) and the feature classes (FCs; L = Linear, Q = Quadratic, P = Product, T = Threshold, H = Hinge) and (3) remove variables that did not have significant contribution to the model. We tested for RM = 0.5-10, at a step of 0.5, while gradually removing FCs. Model selection was based on the average test area under the curve value (aveAUC ± replicates' standard deviation) of 100 bootstrap replicates, testing model efficiency on 2,000 points of pseudo-absence randomly selected from the background (or 500 points for clusters distributed in restricted areas, namely Cyclades). Of the original non-correlated variables, only the ones showing a contribution of  $\geq 10\%$  were considered important and were retained in final model-building. The parameters and variables that were used for building the final models are shown in the table. The percentage of contribution shown for each variable is average over replicate runs.

Variable	Variable Definition	Model Fitting	Cyclades	Asia	South	North	South + Asia	ALL
		Points before thinning	42	56	294	222	350	614
		Points after thinning	20	47	210	154	257	431
		RM	2	1.5	1.5	1	1	1
		FCs	LQP	LQP	LQ	LQP	LQP	LQP
		av.AUC	0.890	0.899	0.850	0.895	0.875	0.867
		$\pm$ sd	0.001	0.012	0.005	0.004	0.006	0.004
	Bioclimatic							
bio2	Mean Diurnal Range		42.1	0.2	2.1	2.7	4.6	5.4
	(Mean of monthly (max terr	p-min temp))						
bio3	Isothermality (BIO2/BIO7)(×100)		0.2	8.1	0.2	3.3	3.9	2.4
bio4	Temperature Seasonality (Standard Deviation)		6.4	17	21.4	7.7	22.1	49.7
bio6	Min Temperature of Coldest Month		16.5	3.4	11.5	5.5	12.4	12.4
bio8	Mean Temperature of Wettest Quarter		0.3	37.6	0.5	5.9	6.3	2.4
bio9	Mean Temperature of Driest Quarter		0.8	4.7	49.6	2.5	28.6	4.4
bio12	Annual Precipitation		0.6	0.9	0.3	24.9	1.2	5.2
bio15	Precipitation Seasonality (C	oefficient of Variation)	27.7	17.5	7.0	3.8	8.2	5.6
bio17	Precipitation of Driest Quar	ter	5.4	1.9	4.0	34.5	7.0	3.1
	Topographical							
slope	Max Rate of Elevation Char Surroundings	nge between Location and	-	10.7	3.5	9.4	5.8	9.3

**Table S6.** Top: Estimates of Schoener's D (Schoener, 1968) and Hellinger's I (Hellinger, 1909), calculated with ENMtools among *Vipera ammodytes* populations divided in five groups, i.e., North, South, Asia, Cyclades and South + Asia. Values of D are given below the diagonal and of I above the diagonal in italics. These tests utilised the environmental information derived from each group's geographical distribution (geographic G-space).

Bottom: Results of the Niche Overlap (NOT) and Niche Divergence (NDT) tests performed in "humbodlt", between pairs of *Vipera ammodytes* groups that returned significant niche similarity in the previous tests. We used only variables contributing >10% in the model and rarified matrices to avoid clustering of points (at a minimum distance of 5 km, thus excluding Cyclades from the test). E-analog gives the percentage of the analogous climate space (environmental E-space), incorporating the spatial distribution of analogous environments of the two groups under comparison. D<sub>obs</sub> corresponds to the Niche Similarity Index quantified with Schoener's D measurement for the E-analog space alone. E<sub>obs</sub> is the observed significance of the Equivalence statistic (in parenthesis), and B are the respective significance of the pairs of Background statistics (1→2 and 2→1), as described in Brown & Carvanal (2019). Tests were either significant at p < 0.05 (\*) or <0.001 (\*\*) or non significant (NS).

ENMtools						
Ι						
D	South	Asia	Cyclades	North	South+Asia	
South		0.877	0.514	0.588	-	
Asia	0.652		0.312	0.754	-	
Cyclades	0.230	0.109*		0.200	0.512	
North	0.309	0.461	0.068*		0.611	
South+Asia	-	-	0.224	0.322		
"humbodlt"						
	Test / E-analog (%)	D <sub>obs</sub>	E <sub>obs</sub>	$B_{2\rightarrow 1}$	$B_{1\rightarrow 2}$	
South-North	<b>NOT</b> / 30.02	0.130	* (0.0297)	* (0.01176)	** (0.0099)	
	<b>NDT</b> / 27.94	0.113	NS (1)	NS (0.25000)	NS (0.2277)	
South+Asia-North	<b>NOT</b> / 44.052	0.171	NS (0.9108)	* (0.01219)	* (0.0104)	
	<b>NDT</b> / 37.51	0.159	NS (0.5148)	* (0.0476)	* (0.0109)	

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