



Short Communication

Phylogenomic insights into the diversity and evolution of Palearctic vipers

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ABSTRACT

Despite decades of molecular research, phylogenetic relationships in Palearctic vipers (genus *Vipera*) still essentially rely on a few loci, such as mitochondrial barcoding genes. Here we examined the diversity and evolution of *Vipera* with ddRAD-seq data from 33 representative species and subspecies. Phylogenomic analyses of ~ 1.1 Mb recovered nine major clades corresponding to known species/species complexes which are generally consistent with the mitochondrial phylogeny, albeit with a few deep discrepancies that highlight past hybridization events. The most spectacular case is the Italian-endemic *V. walser*, which is grouped with the alpine genetic diversity of *V. berus* in the nuclear tree despite carrying a divergent mitogenome related to the Caucasian *V. kaznakovi* complex. Clustering analyses of SNPs suggest potential admixture between diverged Iberian taxa (*V. aspis zinnikeri* and *V. seoanei*), and confirm that the Anatolian *V. pontica* corresponds to occasional hybrids between *V. (ammodytes) meridionalis* and *V. kaznakovi*. Finally, all analyzed lineages of the *V. berus* complex (including *V. walser* and *V. barani*) form vast areas of admixture and may be delimited as subspecies. Our study

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sets grounds for future taxonomic and phylogeographic surveys on Palearctic vipers, a group of prime interest for toxinological, ecological, biogeographic and conservation research.

1. Introduction

By challenging well-established phylogenies based on a few genes, especially mitochondrial DNA (mtDNA) genes, genomic analyses have allowed the most significant leap forward in the fields of ecology, evolution and systematics for decades (Lexer et al., 2013; Dufresnes et al., 2023). In Palearctic amphibians and reptiles, the implementation of high-throughput sequencing in phylogeography has demonstrated a prominent role of hybridization in the diversification of species complexes through hybrid speciation (e.g., Dufresnes et al., 2019; Zinenko et al., 2016), pervasive and potentially adaptive introgression (e.g., Yang et al., 2021), which can blur molecular-based species identification and delimitation (Unmack et al., 2022; Dufresnes et al., 2023), especially when these processes generate mitonuclear discordance (e.g., Gvoždík et al., 2023). Revisiting evolutionary and taxonomic hypotheses with genomic methods such as double-digest Restriction-Associated DNA (ddRAD-seq) has therefore become a standard in herpetological research (e.g., Dufresnes et al., 2019; Gvoždík et al., 2023; Thanou et al., 2023; Mochales-Riño et al., 2023).

With currently ~ 20 recognized species, the genus *Vipera* (Squamata: Viperidae) is the most diverse group of snakes inhabiting the Western Palearctic (Alencar et al., 2016). Like most vipers, all *Vipera* species are viviparous and venomous, and many are globally threatened by human pressures, including persecution and habitat destruction (IUCN, 2024). Palearctic vipers have always fascinated scientists, initially for their toxicity and more recently to address various ecological and evolutionary questions pertaining to the biogeography of the Palearctic realm (e.g., Ursenbacher et al., 2006, 2008; Lucchini et al., 2023), the evolution of reproductive modes (e.g., Fenwick et al., 2012), and the role of hybridization in speciation (Zinenko et al., 2016).

Despite a strong empirical focus, the taxonomic and phylogeographic diversity of *Vipera* remain difficult to reconcile. On the one hand, species and subspecies show great external variability in the traits traditionally assessed in snake taxonomy (e.g., skull morphology, scalation, color patterns; Freitas et al., 2020), but which can be influenced by unsuspected hybridization or local selection alongside many extrinsic factors (e.g., Dubey et al., 2015; Martínez-Freiría et al., 2020b; Mebert et al., 2015, 2017). On the other hand, phylogeographic analyses have essentially relied on mtDNA (e.g., Ursenbacher et al., 2006, 2008; Martínez-Freiría et al., 2020a), together with a few conserved and thus weakly informative nuclear genes (e.g., Alencar et al., 2016; Mizsei et al., 2017; Doniol-Valcroze et al., 2021). While these markers presently offer the most comprehensive reference for species delimitation in the genus (reviewed by Freitas et al., 2020), to what extent the mitochondrial tree depicts the complete evolutionary history of *Vipera* is questionable, especially since many Viperidae species are known to hybridize despite strong molecular and phenotypic divergence (Guiller et al., 2017; Mochales-Riño et al., 2023). Multilocus population genetic/genomic surveys recently conducted on a few species showcased mitonuclear discordances linked to cryptic lineage divergence (e.g., *V. ammodytes*, Thanou et al., 2023), lineage fusion (e.g., *V. ursinii*, Vörös et al., 2022), and even hybrid taxa (e.g., *V. orlovi*; Zinenko et al., 2016). Genomic surveys become particularly helpful to improve taxonomic accuracy, notably to delimit species and subspecies, on which conservation plans and legal protection depend (Dufresnes et al., 2023). Recent ddRAD-seq analyses in the *V. ammodytes* complex suggested to elevate *V. a. meridionalis* as a distinct species (*V. meridionalis*) based on its deep divergence and seemingly lack of gene flow (amid sampling gaps in parapatric ranges), while *V. transcaucasiana* was relegated to subspecies level (as *V. meridionalis transcaucasiana*) for the opposite reasons (Thanou et al., 2023).

A phylogenomic view of the diversification of the whole genus *Vipera* thus appears overdue, especially to reassess the evolutionary significance and phylogenetic placement of taxa for which nuclear data remain absent or inconclusive. In this respect, the most controversial species is perhaps the recently described *V. walser* from the southern Alps (Ghielmi et al., 2016; Doniol-Valcroze et al., 2021). Previously confounded with the adder *V. berus*, *V. walser* was distinguished essentially based on its divergent mitogenome of Caucasian affinity (Ghielmi et al., 2016) and remains unrecognized by taxonomic authorities (Speybroeck et al., 2020) given the weak divergence at the few nuclear genes analyzed (Doniol-Valcroze et al., 2021).

In this study, we report on ddRAD-seq analyses of a selection of samples covering a large part of the phylogeographic and taxonomic diversity of Palearctic vipers of the genus *Vipera* (including subgenus *Pelias*), with a particular focus on European populations of *V. berus* to elucidate the case of *V. walser*. We provide a robust phylogenomic tree for the whole *Vipera* genus and pinpoint major departures from the well-established mitochondrial phylogeny on which the current taxonomy is based (Freitas et al., 2020). Furthermore, we screen for admixture between and within major clades in a provisional attempt to detect signs of reticulate evolution and propose some preliminary taxonomic revisions.

2. Methods

2.1. ddRAD-seq library preparation, sequencing and bioinformatics

Eighty-five tissue samples representing 33 taxa from 17 species (Freitas et al., 2020) were included, originating from known populations and a few recently discovered ones, and identified based on morphology, geographic distribution, as well as previous genetic analyses (File S1). Missing species include *V. graeca*; *V. dinniki*, *V. orlovi*, *V. olguni* and *V. magnifica* (*V. kaznakovi* complex); *V. lotievi*, *V. shemakhensis* and *V. ebnari* (*V. renardi* complex); noting that the validity of some of these species is ambiguous (Freitas et al., 2020). DNA was isolated using the Qiagen Blood and Tissue kit. A ddRAD-seq library was prepared using a customized version of the protocol by Brelsford et al. (2016), which involves enzyme restriction by *MseI* and *SbfI* and a size selection window of 400–500 bp (<https://dx.doi.org/10.17504/protocols.io.kxygx3nzwg8j/v1>). The library was sequenced single-end on an Illumina Next-Seq 550 using the 1 × 150 bp kit.

Bioinformatics steps were conducted with Stacks (Catchen et al., 2013). Raw reads were demultiplexed (function *process_radtags*) and assembled de novo using the dedicated pipeline (*denovo.pl*) with default settings ($-n -m -M$). The obtained catalog contained 1,608,679 loci with a mean effective per-sample coverage of $79.1 \times$. Datasets used in downstream analyses were obtained (function *populations*) using the provided filters.

2.2. Phylogenetic analyses

For phylogenetic analyses, we first considered a concatenated alignment of 7,333 RAD tags (1.1 Mb, 69,630 SNPs) present in at least 60/85 samples ($-p 60 -phytip-var-all$). The alignment was transformed in fasta format using an online converter (https://www.hiv.lanl.gov/content/sequence/FORMAT_CONVERSION/form.html) and a maximum-likelihood tree was built with the web version of IQtree 1.6 (Trifonopoulos et al., 2016). The model finder tool was used to find and apply the best model of sequence evolution. Node support was assessed by 1,000 ultrafast bootstraps. In the absence of non-*Vipera* taxa, we rooted the tree with the *V. ammodytes* complex, which is the first *Vipera* clade to split in the phylogeny of Viperidae (Alencar et al., 2016; Šmíd and

Tolley, 2019; Freitas et al., 2020). Because a few of our samples were flagged as admixed (see below) and can potentially bias the tree topology, we performed a second IQtree analysis without these samples, namely on a concatenated alignment of 3,462 RAD tags (500 kb, 32,561 SNPs) present in at least 60/73 “pure” samples ($-p\ 60$ *-phytip-var-all*) (File S1). The filtering scheme was designed to balance between alignment length while avoiding high proportions of missing data, as a prerequisite for robust ddRAD-seq phylogenies (Ambu et al., 2023).

2.3. Population structure analyses

To explore general ancestry patterns across *Vipera*, we considered a matrix of 698 SNPs present in 80/85 samples ($-p\ 80$ *-write-random-snp -structure*). The dataset was analyzed with STRUCTURE 2.3 (Pritchard et al., 2000), using the admixture model and correlated allele frequencies. To facilitate convergence and avoid “ghost clusters”, we opted for short runs (1,000 iterations) after longer burn-in periods (20,000 iterations), which proved efficient to obtain explicit clustering solutions

with better likelihood in high K values. In addition, clustering programs have known biases when analyzing multiple genetically differentiated populations with low sample sizes. To verify that the few instances of shared ancestry recovered are genuine (see Results), we also ran $K = 2$ clustering analyses on the three ad hoc SNP matrices obtained for each candidate admixed and parental samples: (1) 400 SNPs present in our 16 samples of *V. seoanei* and *V. latastei-monticola* ($-p\ 16$ *-write-random-snp -structure*); (2) 2,590 SNPs present in our 14 samples of *V. seoanei* and *V. aspis* ($-p\ 14$ *-write-random-snp -structure*); (3) 2,118 SNPs present in our 9 samples of the *V. ammodytes* and *V. kaznakovi* complexes, including *V. pontica* ($-p\ 9$ *-write-random-snp -structure*). Finally, to infer genetic structure in the *V. berus* complex, we considered a matrix of 415 SNPs present in all 30 samples ($-p\ 30$ *-write-random-snp -structure*). The dataset was analyzed with STRUCTURE as above, as well as with a Principal Component Analysis (PCA) using the *adeigenet* R package (Jombart, 2008). The stringent filtering parameters were specifically chosen to minimize or avoid any missing data ($-p = n$) and to include a single SNP per RAD tag, which is more appropriate for the downstream

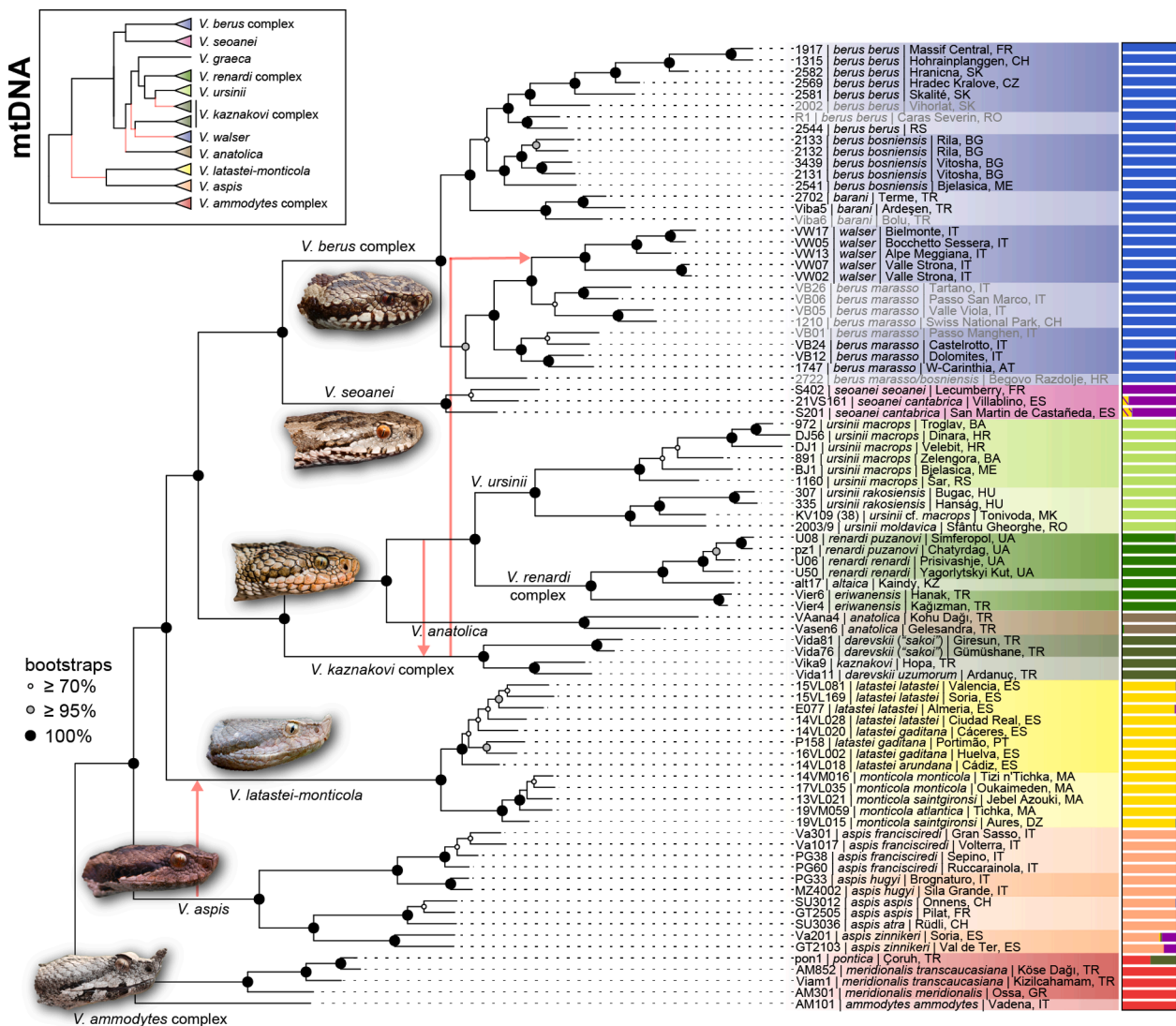


Fig. 1. Maximum-likelihood tree of *Vipera* based on 1.1 Mb (7,333 concatenated RAD tags) and ancestry to nine identified clusters based on 698 SNPs (presumably unlinked). Circles show node support based on 1,000 bootstrap replicates; red arrows highlight mitochondrial introgression according to the major mitonuclear discordances identified by comparing our tree with the mtDNA tree of Freitas et al. (2020), schematized in inset (discordant branches in red); color shades distinguish the main lineages within clades; grey labels emphasize admixed samples within the *V. berus* complex (see Fig. 2). Subset analyses (File S3) support the shared ancestries retrieved in *V. aspis zinnikeri* and *V. pontica*, but not in *V. seoanei cantabrica* (represented by dash lines). Photos (top to bottom): *V. berus walser* (SG), *V. seoanei* (FMF), *V. erivanensis* (DJA), *V. monticola* (FMF), *V. aspis* (SU), *V. ammodytes* (BH). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

analyses.

3. Results

3.1. Phylogenetic analyses

The IQtree for all 85 samples (~1.1 Mb, 7,333 RAD tags) uses TIM3 + F + R2 as the best-fit model according to the BIC criterion. It features nine robustly-supported major clades that correspond to the following species/species complexes (Fig. 1): (1) the *V. ammodytes* complex, including *V. meridionalis-transcaucasiana* and *V. pontica* from West Asia; (2) *V. aspis*; (3) *V. latastei-monticola*; (4–7) four related clades corresponding to the *V. kaznakovi* complex (including *V. darevskii* “sakai”), *V. anatolica*, the *V. renardi* complex (including *V. altaica* and *V. erivanensis*), and *V. ursinii*; (8) *V. seoanei*; (9) the *V. berus* complex (including *V. barani* and *V. walser*). The same topology is retrieved when analyzing only the 73 samples free of putative admixture (File S2; see below).

Our ddRAD-seq tree features a few noticeable discordances from the mitochondrial phylogeny (Fig. 1). First, *V. walser* is grouped with the *V. berus* complex, as the sister lineage of *V. berus marasso*, while it is the sister lineage of the *V. kaznakovi* complex in the mtDNA tree. Second, *V. aspis* and *V. latastei-monticola* are polyphyletic, while they are retrieved as sister clades in the mtDNA tree. Third, the phylogenetic position of the *V. kaznakovi* complex and *V. anatolica* differ between the ddRAD-seq and mtDNA analyses.

Furthermore, several currently recognized species and subspecies (following Uetz et al., 2024; see also Freitas et al., 2020) appear weakly differentiated from their closest relative and/or are not robustly retrieved as separate lineages (Fig. 1, File S2). This includes *V. walser*, *V. barani*, *V. seoanei cantabrica*, *V. ursinii macrops* from the Bistra Mountains (western North Macedonia), *V. darevskii uzumorum*, *V. latastei gaditana*, *V. monticola saintgironi*, *V. monticola atlantica*, *V. pontica*.

3.2. Population structure analyses

Bayesian clustering analyses of the 85 samples (698 SNPs) recovered the nine clades as separate genetic groups (Fig. 1). A few samples

received intermediate ancestry coefficients: *V. seoanei cantabrica* features ancestry with the *V. latastei/monticola* cluster, *V. aspis zinnikeri* features ancestry with the *V. seoanei* cluster, and our *V. pontica* sample is a mix between the clusters attributed to the *V. ammodytes* and *V. kaznakovi* complexes. The patterns hold for *V. aspis zinnikeri* and *V. pontica* in the subset analyses, but not for *V. seoanei cantabrica*, which is instead assigned only to the *V. seoanei* cluster (File S3). The corresponding samples do not show fewer reads or higher proportions of missing data compared to the rest of the dataset (File S1).

Finally, Bayesian clustering analyses of the *V. berus* complex dataset (415 SNPs) distinguished up to seven genetic groups (Fig. 2), all matching the lineages identified by the phylogeny: (1–3) *V. b. berus* from W-Europe, NE-Europe, and the Carpathian region; (4) *V. berus bosniensis* from the Balkans; (5) *V. berus marasso* from the eastern Alps; (6) *V. walser* from the Central Alps; (7) *V. barani* from Anatolia (Fig. 2). As further shown by the PCA, the genetic structure follows geographic gradients, and admixture is retrieved between all clusters across relatively large distances (Fig. 2). Admixed samples are placed at intermediate positions in the tree (Fig. 1).

4. Discussion

Based on genome-wide data, we provide a preliminary overview of the nuclear diversity and phylogeny of Palearctic vipers of the genus *Vipera*. Our phylogeny supports the results obtained by ddRAD-seq analyses on particular species sets (Zinenko et al., 2016; Thanou et al., 2023), and broadly corresponds to the mitochondrial phylogeny (Freitas et al., 2020). However, the deep mitonuclear discordances and the nuclear admixture documented in several samples illustrate the need to re-evaluate the phylogeographic history and systematics of the genus, notably by accounting for hybridization both within and between the major clades.

The few discrepancies between the nuclear and mitochondrial trees (Fig. 1) emphasize past mitochondrial transfers, namely between the ancestors of *V. ursinii/renardi/kaznakovi* and the ancestors of *V. aspis/latastei*. The mitochondrial captures must have occurred soon after the emergence of these old clades, which implies higher opportunities for contact than the current allopatric ranges suggest. The most striking

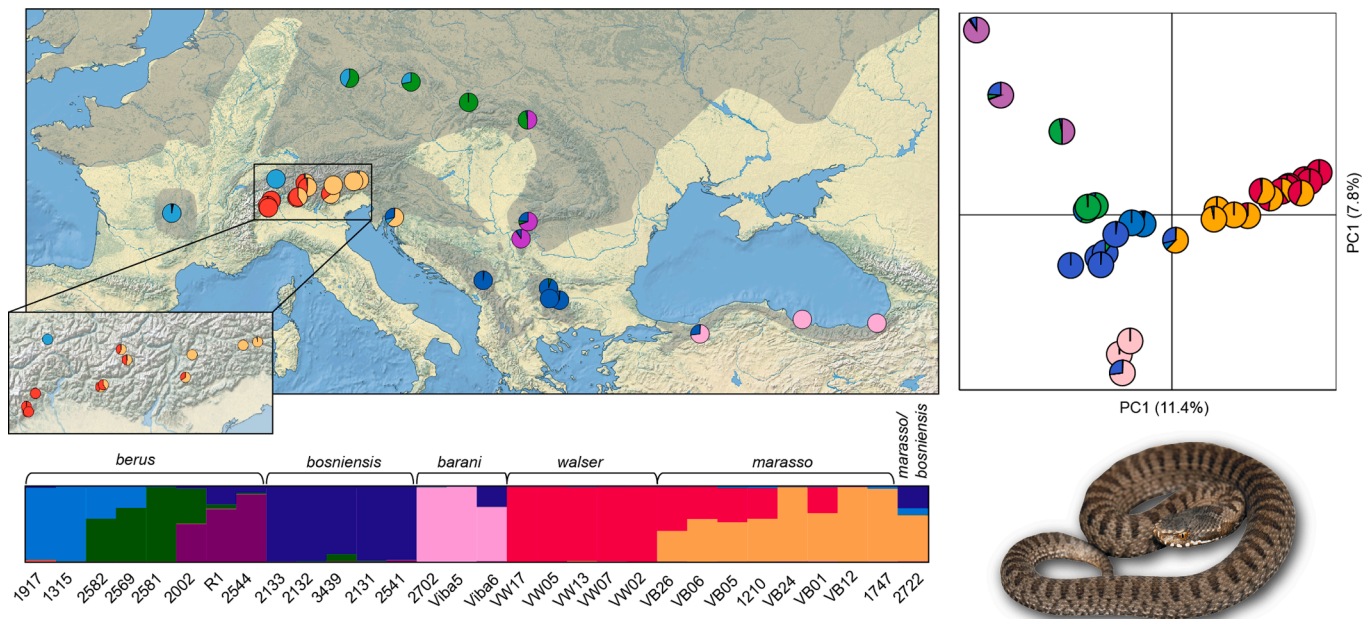


Fig. 2. Genetic structure in the *V. berus* complex, based on 30 samples genotyped for 415 (presumably unlinked) SNPs, as investigated by clustering analyses (left) and a PCA on allele frequency (right). Barplots and pie charts show individual ancestry to the seven groups recovered by Bayesian clustering. Photo: *V. berus bosniensis* (DJa).

mitonuclear discordance involves the subalpine *V. walser*. In the nuclear genome, these populations belong to the alpine diversity of the adder *V. berus* (as the sister lineage of geographically proximate *V. b. marasso*; Fig. 1), despite carrying mtDNA related to the Caucasian *V. kaznakovi* complex (Ghielmi et al., 2016). This result confirms previous suspicions regarding the conspecificity of *V. walser* and *V. berus* following the analysis of additional nuclear genes (Doniol-Volcroze et al., 2021) since the original description (Ghielmi et al., 2016). Given that the nuclear differentiation of *V. walser* appears much weaker than its mitochondrial divergence from any extant lineage, two possible scenarios can be envisaged (see also Doniol-Volcroze et al., 2021). The populations of *V. walser* may have once represented an old subalpine endemic lineage of eastern affinities – relic of a much wider distribution of the *V. kaznakovi* ancestors across Europe – which was subsequently assimilated by surrounding *V. berus* through nuclear introgression. Alternatively, the southern Alps were only inhabited by *V. berus* ancestors, and the diverged *V. walser* matriline would have been acquired through a relatively recent hybridization event with an unsampled (probably extinct) species from the *V. kaznakovi* clade. Either way, this intricate situation supports unexpected biogeographic connections between populations that are now separated by thousands of kilometers (Doniol-Volcroze et al., 2021) and highlights the dynamic range shifts experienced by Palearctic vipers following the dramatic climatic and environmental changes in Europe within the last millions of years (Lucchini et al., 2023).

In NE-Anatolia, the mixed genetic composition of *V. pontica* corroborates suspicions that this enigmatic taxon (known from only two specimens) represents sporadic hybrids between the sympatric *V. meridionalis transcaucasiana* and *V. kaznakovi* (Freitas et al., 2020 and references therein). Accordingly, occasional hybridization (and gene flow) between many pairs of diverged *Vipera* species has been well documented in their area of parapatry or sympatry (Tarroso et al., 2014; Mebert et al., 2015; Zinenko et al., 2016; Guiller et al., 2017). Additionally, the low *V. seoanei* ancestry in *V. aspis zinnikeri* could reflect the reticulate evolution of these taxa, or indicate that our samples encompassed an unsuspected contact zone (see also Tarroso et al., 2014). Alternatively, we cannot rule out an artifact in both clustering datasets, as for *V. seoanei cantabrica* in the genus-wide analysis (File S4). Anyhow, the fact that palearctic vipers retain the capacity to sire offspring along their wide spectrum of evolutionary divergence – the origin of *Vipera* was dated to ~ 15 Mya (Alencar et al., 2016; Šmíd and Tolley, 2019; Freitas et al., 2020) – offers fascinating prospects to study the role of hybridization as a driver of their rapid diversification (Zinenko et al., 2016), as well as to generate their remarkable variability of color patterns (e.g., Martínez-Freiría et al., 2020b), as seen e.g., in *Podarcis* lizards (Yang et al., 2021).

The genomic structure retrieved in the Central European and Anatolian populations of the *V. berus* complex coincides with the mitochondrial phylogeography of this species (Ursenbacher et al., 2006). The lineages identified seem to admix across wide distances, which *a priori* suggests little reproductive barriers. Therefore, if one agrees to delimit species and subspecies as evolutionary lineages that differ in their capacity to hybridize and mix their genomes (Dufresnes et al., 2023), the whole *V. berus* clade may be regarded as a single species. Some of the taxa presently listed as species may thus be ranked down as subspecies (e.g., *V. berus walser*, *V. berus barani*), and reciprocally, unnamed lineages may warrant taxonomic attention, noting that some names may already be available (Golay et al., 1993). From a biogeographic perspective, the *V. berus* diversification in Central Europe is reminiscent of the patterns found in other herpetofauna with highland affinities, notably the Alpine newt (e.g., Robbemont et al., 2023).

Other peculiar phylogenomic patterns are worth further investigations. As suspected from mtDNA (Vörös et al., 2022), our sample from the Bistra Mountains, assigned to *V. ursinii macrops*, represents a distinct evolutionary lineage from that subspecies. In Anatolia, the subspecies *V. dorevskii uzumorum* is grouped with *V. kaznakovi* rather

than with samples assigned to *V. dorevskii*. Reciprocally, some *V. seoanei* and *V. latastei-monticola* subspecies appear genetically close to the nominal forms, which could imply synonymy (e.g., *V. s. cantabrica* was suggested as invalid based on mtDNA, Martínez-Freiría et al., 2015), or that the analyzed individuals may have been sampled from intergradation zones.

Our study empirically demonstrates the effectiveness of genome-reduction methods like ddRADseq to obtain reliable phylogenetic insights into animal groups which evolutionary history is potentially intertwined by hybridization. The reported mitonuclear discordance in *V. b. walser* also adds another clear-cut case of the limits of mtDNA-based systematics in animals (Dufresnes and Jablonski, 2022), and calls to avoid concatenating mitochondrial with nuclear gene sequences in phylogenetic trees – as traditionally performed for *Vipera* (e.g., Alencar et al., 2016; Šmíd and Tolley, 2019). In the wait of extensive phylogeographic sampling for all of the identified clades and the species yet unsampled, our study lays the first stone towards a robust genomic framework from which to implement more informed taxonomic revisions and thus better prioritize the conservation of the rich species and subspecies diversity of these fascinating snakes.

CRedit authorship contribution statement

Christophe Dufresnes: Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Samuele Ghielmi:** Writing – review & editing, Resources. **Bálint Halpern:** Writing – review & editing, Resources. **Fernando Martínez-Freiría:** Writing – review & editing, Resources. **Konrad Mebert:** Writing – review & editing, Resources. **Dusan Jelić:** Writing – review & editing, Resources. **Jelka Crnobrnja-Isailović:** Writing – review & editing, Resources. **Sven Gippner:** Writing – review & editing, Methodology. **Daniel Jablonski:** Writing – review & editing, Visualization. **Ulrich Joger:** Writing – review & editing, Resources. **Lorenzo Laddaga:** Writing – review & editing, Resources. **Silviu Petrovan:** Writing – review & editing, Resources. **Ljiljana Tomović:** Writing – review & editing, Resources. **Judit Vörös:** Writing – review & editing, Resources. **Naşit İğci:** Writing – review & editing, Resources. **Mert Kariş:** Writing – review & editing, Resources. **Oleksandr Zinenko:** Writing – review & editing, Resources. **Sylvain Ursenbacher:** Writing – original draft, Resources, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data have been uploaded to public repositories as indicated in the data availability statement

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Appendix A. Supplementary data

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