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Well-known species, unexpected results: high genetic diversity in declining *Vipera ursinii* in central, eastern and southeastern Europe

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Abstract. The Meadow and Steppe viper, *Vipera ursinii-renardi* complex is a well-studied group that is divided into several morphological subspecies. In this study, we combine the analyses of two mitochondrial genes with 9 microsatellite markers to compare both phylogenetic signals. Whereas the signal is similar between both genomes within most subspecies, the relative relationships between subspecies are more differentiated. Moreover, the nuclear phylogenetic reconstruction supports genetic homogeneity within *V. u. macrops* (in contrast to mtDNA). Both genetic portions show an unexpected differentiation between a population from Bistra Mountain and other *V. u. macrops* populations. Globally, the microsatellite markers suggest high genetic diversity in most subspecies, even in *V. u. rakosiensis* which is highly threatened; only *V. u. macrops* showed a limited genetic diversity. Within lowland subspecies, the differentiation between populations is globally limited compared to the distance between them (except in some populations of *V. u. moldavica*). The limited differentiation might be the consequence of a recent isolation (few decades) of previously large populations. Nevertheless, the only way to maintain this genetic diversity and to avoid an increase in genetic differentiation between populations in the future is to recreate suitable habitats and reconnect the populations.

Keywords: cytonuclear discordance, meadow vipers, microsatellite markers, mitochondrial DNA, population genetics, Viperidae.

Introduction

For several decades, the use of DNA, especially mtDNA, allowed us to disentangle phylogenetic relationships between populations and species, being able to strongly improve the knowledge on past history of species (Avise, 2004). Although the limits of mtDNA analyses are known for a long time (e.g., maternal inheritance, thus lack of detection of hybrids), the sequencing of several parts of this genome helped to identify evolutionary units formed in the course of dispersion and evolution. Further, if confirmed by other lines of evidence (e.g., morphological differences, indication of genetic isolation and divergence across nuclear genome) have led to taxonomic conclusions (Mizsei et al., 2017; Speybroeck et al., 2020). But this approach based on a single locus is more and more criticised to define taxa, as numerous discrepancies in the stories they tell (e.g., Ujvari et al., 2005; Edwards and Bensch, 2009). Indeed, the more frequent use of nuclear markers at the phylogenetic level demonstrated numerous cases of contradiction between historical reconstruction resulting from both genomes (Toews and Brelsford, 2012). To analyse nDNA, several introns are frequently used in vertebrates, but they often demonstrate a limited power of distinction between taxa compared to mtDNA (Freitas et al., 2020). Other highly variable markers like microsatellites (also known as SSR for simple sequence repeat) or AFLP have also been used for phylogenetic studies, but their high level of variation only allows the investigation of the recent history of species (Alvarez et al., 2012; Garcia et al., 2012).

Several examples of contradiction between both mtDNA and nDNA genomes have been recently highlighted in European herpetofauna, for instance in the green toad (*Bufotes viridis*, Dufresnes et al., 2018), in the Macedonian crested newt (*Triturus macedonicus*) in the Balkan Peninsula (Wielstra and Arntzen, 2020), or in the fire salamander (*Salamandra salamandra*) in Italy (Bisconti et al., 2018). The case of *Vipera walser* is also emblematic: whereas mtDNA and a single nDNA gene analyses by Ghielmi et al. (2016) suggested an astonishing genetic history of the "adder" of northwestern Italy with an old isolation and affinities with Caucasus viper species, the combination of 5 introns suggested a high level of introgression between *V. walser* and *V. berus* (Doniol-Valcroze et al., 2021), even if the introns used have been suggested to have limited phylogenetic signal in Eurasian vipers (Freitas et al., 2020). This last example, as well as other similar examples in the Caucasus region (see

Zinenko et al., 2016) demonstrate complex history of Eurasian vipers, probably due to recent introgression events.

The Meadow and Steppe vipers, *Vipera ursinii-renardi* complex, were divided into several morphological subspecies. Numerous studies were conducted during the last decade, including only mtDNA genes (Ferchaud et al., 2012; Gvozdik et al., 2012; Zinenko et al., 2015). All demonstrated similar phylogenetic history, with a strong support for most subspecies previously described (*V. u. ursinii* in France and Italy; *V. u. rakosiensis* in Hungary and western Romania; *V. u. moldavica* in eastern Romania and Republic of Moldova; *V. u. greaca* in Greece and Albania – now recognised as a species [Mizsei et al., 2017]). However, these studies highlighted a polyphyletic position of *V. u. macrops*, with populations of northwestern Dinarides (Croatia and western part of Bosnia and Herzegovina) being more related to *V. u. ursinii* and populations from southeastern Dinarides (southeastern part of Bosnia and Herzegovina, Montenegro, Serbia, western part of Kosovo province) and Hellenides (eastern part of Kosovo province and North Macedonia) being more related to *V. u. rakosiensis* and *V. u. moldavica* (Ferchaud et al., 2012; Gvozdik et al., 2012; Zinenko et al., 2015). However, a close inspection of the article of Mizsei et al. (2017) reveals that the nDNA of northwestern and southeastern populations of *V. u. macrops* are more similar compared to other *V. ursinii* subspecies, also suggesting some discrepancies between nDNA and mtDNA within *V. ursinii*.

The Meadow viper (*Vipera ursinii*) is considered as one of the most threatened snake species in Europe (Cox and Temple, 2009), mainly due to habitat loss and fragmentation of lowland populations, natural fragmentation of mountainous taxa, overgrowth of mountain meadows or, on the opposite, overgrazing. For instance, several populations of *V. u. rakosiensis* in the Danube plains and surroundings have already disappeared (e.g., all populations in Austria; Nilson and Andren, 1997) or are reduced to a very tiny part of the historical distribution range, due to habitat destruction in order to create crop fields or by planting trees and active culling (Péchy et al., 2015). Consequently, these alterations led to a strongly fragmented distribution, where individuals could not move from one remnant population to another. This strong isolation could also have an impact on genetic diversity. Moreover, it has been shown that Eurasian vipers are particularly sensitive to lack of genetic diversity: in Sweden, a *V. berus* population was decreasing in population size and adult recruitment until additional genetic variability was artificially added by reintroducing new males (Madsen et al., 1996, 1999, 2004). Consequently, vipers seem particularly sensitive to low genetic heterogeneity, and thus evaluating this diversity in small and isolated populations of *V. ursinii*, especially in the context of current habitat improvement thanks to several LIFE projects in Hungary and Romania (Péchy et al., 2015) is important. Indeed, if high genetic diversity has been maintained in the remnant populations, the bottleneck effect would have been reduced and thus the chance of survival should be higher. On the contrary, if genetic diversity is restricted compared to more natural populations, human translocation may be considered, keeping in mind the risk of adverse effects of outbreeding or the introduction of lethal alleles to genetically impoverished, but purged from genetic load populations (Madsen et al., 1999, 2004). The use of highly polymorphic markers, like microsatellite markers, is, in this case, an adapted method to investigate genetic diversity within populations and regions of the different subspecies of *V. ursinii*. Moreover, the use of these markers has been shown to provide valuable information on more recent historical (re)colonisation within Eurasian vipers (Ursenbacher et al., 2015).

To investigate the genetic diversity within *V. ursinii* in Central, Eastern and Southeastern Europe, we performed a large sampling of several known populations of *V. u. rakosiensis*, *V. u. macrops* and *V. u. moldavica*. We also added

samples of *V. renardi* geographically close to the sister species, to explore and compare diversity between them. The aims were to examine both mtDNA (for which the phylogenetic reconstruction seems relatively stable between all previous studies) and nDNA (using microsatellite markers). Thus we aimed to i) replicate the mtDNA analyses with two markers to confirm previous results but including several new locations in Central, Eastern and Southeastern Europe; ii) determine the geographic delimitation between both lineages occurring within *V. u. macrops*; iii) test if nDNA confirms the phylogenetic signal obtained with mtDNA only; iv) evaluate the genetic diversity within the different regions and subspecies to define if diversity is related to current strong population isolation or if it is more historically grounded; and finally v) focus on the genetic structure within the largely sampled regions within *V. u. rakosensis* and *V. u. moldavica*. By evaluating and comparing genetic diversity between regions and subspecies, their level of genetic differentiation and their historical relationships, results should be of great importance to understand the genetic structure of this species, but should also yield some new insights for the conservation of *V. ursinii*.

Material and methods

Sampling

Tissue samples were collected by tail or scale clipping, from shed skin or by obtaining blood from 359 living animals or carcasses from 11 regions (44 sites) in three subspecies of *V. ursinii* (137 *V. ursinii rakosiensis* from 3 regions [7 sites], 61 *V. u. moldavica* from 2 regions [4 sites], 111 *V. u. macrops* from 3 regions [17 sites]) and from 50 individuals (3 regions, 16 sites) of *V. renardi* (fig. 1, supplementary table S1). With this sampling we aimed to cover the distribution range of the subspecies/species as much as possible. Tissue samples were preserved in 96% ethanol (scale or tail clip), or liquid nitrogen (blood) and were kept at −80°C until processing in the Collection of Genetic Resources of the Laboratory of Molecular Taxonomy in the Hungarian Natural History Museum. Shed skins were placed in a sterile zip lock bag with silica gel beads and kept at 18°C registered in the same collection. Moreover, 27 mtDNA sequences were obtained from 37 additional samples from different regions and subspecies: 27 samples of *V. u. macrops* from 14 localities, 6 samples from *V. u. moldavica* from 2 localities, 3 *V. u. rakosiensis* from 1 locality and 1 *V. renardi* (see supplementary tables S1 and S2). Finally, mtDNA sequences of *V. ursinii rakosiensis* (6), *V. u. moldavica* (3), *V. u. macrops* (1), *V. u. ursinii* (2) and *V. renardi* (1) from additional locations analysed by Ferchaud et al. (2012) downloaded from NCBI Genbank were used for complementing our dataset with additional haplotypes (supplementary table S1). In addition, one sample of *V. graeca* (Greece) and one of *V. berus* (*V. berus berus*; Switzerland) were used as outgroups for the mtDNA analyses.

Laboratory analyses

Whole genomic DNA was extracted either using DNeasy Blood&Tissue Kit (tails) or QIAmp DNA Mini Kit (swabs) extraction kits following the manufacturer's protocols (Qiagen, Hilden, Germany). Two mitochondrial gene regions were amplified by PCR, on which a 1116 bp long partial sequence of the mitochondrial cytochrome b gene (cytb), using the primer pairs L14724Vb – H15914Vb (Ursenbacher et al., 2006) and a 804 bp long fragment of the mitochondrial NADH dehydrogenase 4 gene (ND4) using the primer pairs ND4-H12763 (Arévalo et al., 1994) were sequenced. PCRs were performed in a total volume of 25 μl using 1 U of Taq polymerase (Thermo Fisher Scientific, Waltham, USA), 2 mM $MgCl_2$, 15 pmol dNTPs (Thermo Fisher Scientific, Waltham, USA), 50 pmol of both primers and ca. 50 ng of genomic DNA with the following profiles: 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 50°C for 60 s and 72°C for 3 min, followed by a 10 min final extension at 72°C. Amplified double strand products were purified by using High Pure PCR Product Purification Kit (Sigma-Aldrich, St. Louis, USA) and directly sequenced from both directions using BigDye Terminator v3.1 Cycle sequencing chemistry on an ABI 3130 Genetic Analyser (Applied Biosystems, Foster City, USA). Consensus sequences were compiled using BioEdit version 7.2.6 (Hall, 1999) and aligned manually.

For all samples mentioned previously (under *Sampling*), we analysed 9 microsatellite loci developed for *V. ursinii* (Vu55, Vu38, Vu58; Metzger et al., 2011) and *V. berus* (Vb-3, Vb-37, Vb-64, Vb-71 from Carlsson et al. 2003; Vb-A11,Vb-D17 from Ursenbacher et al., 2009) and PCR reactions were performed following protocols used in the microsatellite description. Fragment length analysis was run on an Applied Biosystem 3130 Genetic Analyzer under the FragmentAnalysis 50_POP7 protocol. Data collection and scoring were performed with the Software Peak Scanner v1.0 (Applied Biosystems, Foster City, USA).

Statistical analyses at regional scale

mtDNA. After a visual control of the sequences, identical haplotypes were regrouped. Additional sequences from GenBank were included when individuals had been sampled within the investigated regions but not at the exact location as the samples used here. These additional specimens were sequenced for both cytb and ND4. As cytb and ND4 belong

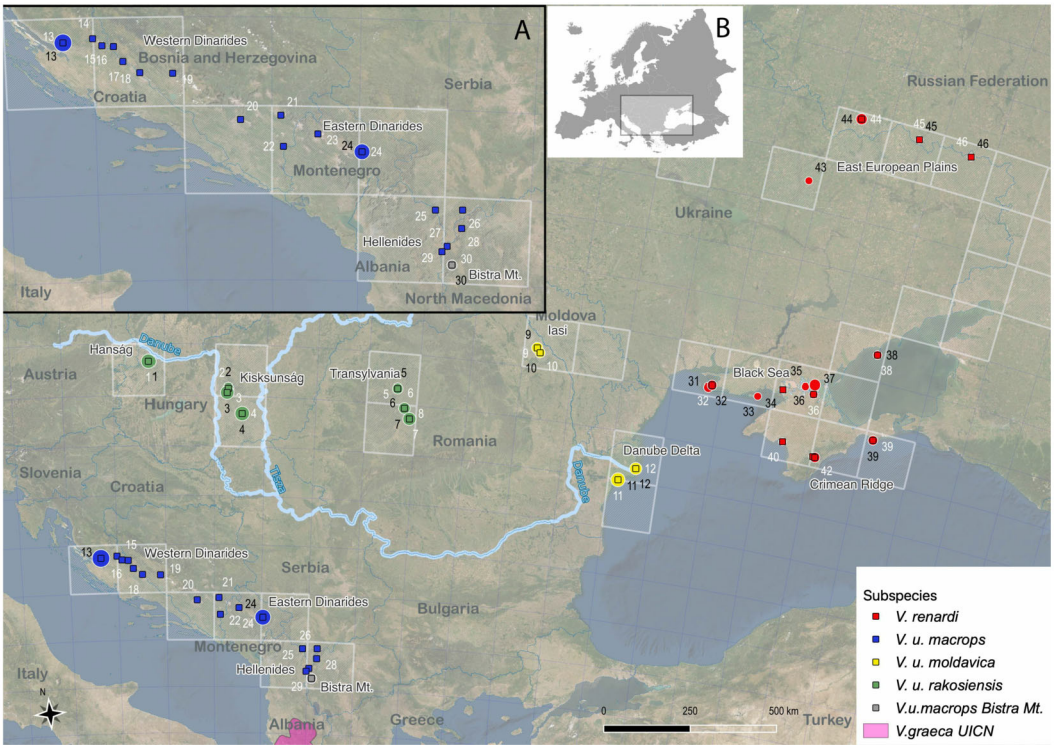

Figure 1. Location of the samples used in the study: squares represent mtDNA data, round symbols represent microsatellites data. The size of the round symbols is proportional to the number of samples used. Locality numbers correspond with supplementary table S1 (in black when microsatellite data are available; in white when mtDNA data). The colours of the marks are different between subspecies: green: *V. ursinii rakosiensis*, yellow: *V. u. moldavica*, blue: *V. u. macrops*, grey: *V. u. macrops* from Bistra Mt., red: *V. renardi*. White striped grids show distribution of each subspecies/species on a 100x100 UTM grid resolution (after Sillero et al., 2014). Distribution area of *V. greaca* (from IUCN red list, Mizsei et al., 2018) is colored in pink. On the top left, insert A shows a zoom in the *V. ursinii macrops* region, while insert B illustrates the location of study area within Europe.

to the same genome (mtDNA), both genes were concatenated for the phylogenetic analyses. The best model evaluation was selected using Mega-X v10.1.8 (Kumar et al., 2018; Stecher et al., 2020). Maximum Likelihood (ML) was investigated using PhyML v3.0 (Guindon et al., 2010) with GTR + I + G model and branch robustness was conducted with 1000 bootstraps, whereas Maximum Parsimony (MP) support was evaluated using PAUP* v4.0a (Swofford, 2002) with 1000 bootstraps. Bayesian Inferences were assessed using MrBayes V3.2 (Ronquist and Huelsenback, 2003) with four Metropolis-coupled Markov chain Monte Carlo (MCMC) and two separate runs of 5 x 10^6 generations sampled every 1000 generations. A burnin of 10% was applied after checking for stability with Tracer 1.6 (Rambaut et al., 2014). Similar analyses (ML, MP, and MrBayes) were also conducted for each gene separately with the methods mentioned above and provided similar topologies compare to both genes concatenated. Moreover, the genetic diversity within taxa was determined with p-distance.

A haplotype network was determined using the TCS network approach implemented in PopART v1.7 (Leigh and Bryant, 2015), using the information of the species and subspecies for colouring the different haplotypes.

nDNA. Linkage disequilibrium between the different microsatellite markers was tested with FSTAT v2.9.3 (Goudet, 1995). We did not look for Hardy Weinberg (HW) disequilibrium as only a few samples had been collected in the same site and that region sampling is indeed the regrouping of individuals from different populations; consequently, the assumption of HW (random mating, no substructure) is not covered. As the detection of null allele is mainly based on the lack of HW equilibrium (see MICROCHECKER; Van Oosterhout et al., 2004), it was also not possible to check for this aspect at the regional level.

Within each region, genetic diversity (observed and expected heterozygosity, respectively H_O and H_E) were calculated using GenAlEx 6.51b2 (Smouse et al., 2017), and allelic richness (Ar) was evaluated with FSTAT. Phylogenetic reconstruction of genetic relationships between regions was calculated using Cavalli-Sforza and Edwards *Dc* distance (Cavalli-Sforza and Edward, 1967) using the software POPULATIONS 1.2.28 (Langella, 1999). The

strength of the branches were tested with 1000 bootstraps. Moreover, a Principal Coordinate Analysis (PCoA) was conducted using Genelax.

Combined nDNA and mtDNA

We compared both mtDNA phylogenetic relationship and nDNA distance tree for the different regions. For the nDNA, the tree calculated with POPULATIONS as mentioned before was used. Mitochondrial DNA Tajima-Nei (Tajima and Nei, 1984) distance matrix was calculated between regions using Mega-X gathering all sequenced haplotypes per region. An UPGMA phylogenetic reconstruction was later conducted with Mega-X, allowing comparison of both trees with CompPhy (Fiorini et al., 2014).

Statistical analyses within V. ursinii *subspecies*

Within *V. u. rakosiensis*, *V. u. moldavica* and *V. u. macrops*, respectively, genetic diversity was compared between all sampled populations. For *V. u. rakosiensis* and *V. u. macrops*, up to 9 microsatellite markers were amplified. For *V. u. moldavica*, only 5 markers used for the analyses at large scale were scored. Hardy-Weinberg equilibrium was tested within each population using GenAlEx. Population genetic diversity H_O, H_E, Ar) and genetic differentiation were calculated with GenAlEx and FSTAT as mentioned above. Genetic structuring was inferred using model-based clustering methods with STRUCTURE 2.3.4 (Pritchard et al., 2000). Best value of K was determined using the Evanno et al. approach (Evanno et al., 2005) with Structure Harvest (Earl and vonHoldt, 2012). Relationships between the different populations were visualised with a PCoA and with a phylogenetic reconstruction of the genetic differences (same method for the comparison between regions) when at least 4 populations were present within a subspecies; moreover isolation by distance (IBD) was also calculated by comparing corrected genetic distance ($F_{ST}/[1 - F_{ST}]$) with the geographical distance (ln[air distance]) following Rousset (1997) using the *mantel.rtest* function of the package ade4 in R (R Core Team, 2021).

Results

mtDNA

The amplification of the cytb provided 1116 bp usable, whereas the amplification of the ND4 provided 804 bp, therefore a total of 1920 bp for the concatenate dataset. The analyses of 105 samples provided 48 different unique haplotypes (both genes combined). Including the 13 haplotypes of *V. ursinii* sensu lato from GenBank as well as two outgroups (*V. beru*s and *V. graeca*) for a total of 63 unique haplotypes (supplementary table S1 and S2), the concatenated alignment presented 270 variable and 171 parsimony-informative sites. The best model of genetic evolution calculated with MEGA was HKY + G + I.

Phylogenetic reconstruction conducted using ML, MP and Bayesian inferences provided congruent trees (fig. 2), with strong support for all main mtDNA groups. Our results confirm the split between *V. ursinii* and *V. renardi*, as well as the affinity of *V. u. macrops* from the Western Dinarides (Croatia, western Bosnia and Herzegovina) with *V. u. ursinii*. *Vipera u. macrops* from the Eastern Dinarides (southern Bosnia and Herzegovina, Montenegro) and Hellenides (eastern Kosovo province and North Macedonia) is recovered as the sister group to the clade formed by *V. u. moldavica* and *V. u. rakosiensis*. It is to note the unexpected position of samples of *V. u. macrops* from Bistra Mt., North Macedonia (Medenica and Tonivoda, two locations separated by 4 km). More precisely, 4 samples (2 haplotypes) from locality Medenica are included into *V. u. moldavica*, whereas one from locality Tonivoda and one from Medenica constitute a highly separated clade within *V. u. rakosiensis*. Contamination is very unlikely, as the haplotypes found in these two locations are not presented in other analysed populations. We also think it is unlikely that we amplified NUMTs (nuclear mitochondrial DNA) as both amplified genes provided similar positions of these samples (data not shown).

Globally, genetic diversity measured with the mtDNA (excluding Bistra Mt., Medenica and Tonivoda) is low within *V. u. rakosiensis* (p-distance = 0.0015; cytb only: p-distance = 0.0017), and *V. u. moldavica* (p-distance = 0.0016; 0.0022), whereas this diversity is higher in *V. u. macrops*, separately from the southeastern part (p-distance = 0.0028; 0.0019) or from the northwestern part (p-distance = 0.0049; 0.0056) or within *V. renardi* in Ukraine and closed Russia (p = 0.0047).

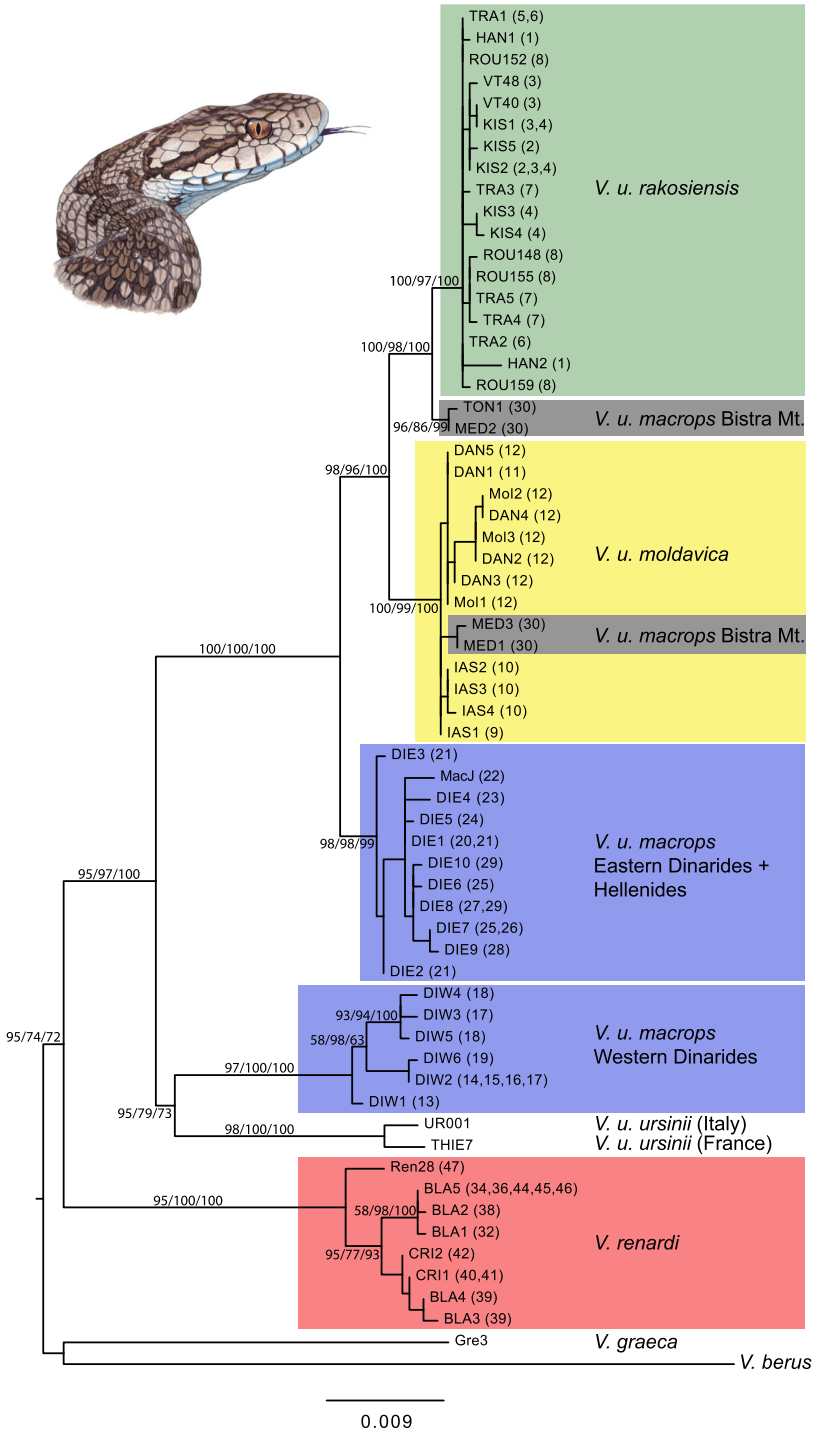

Figure 2. Maximum-likelihood tree from combined data (Cytochrome b and ND4, totalling 1920 bp) for different subspecies of *Vipera ursinii*. Values of bootstrap support for maximum likelihood (first) maximum parsimony (middle) are shown for nodes found in more than 50% of 1000 trees, as well as posterior probability from Bayesian inference (right). The population number (see fig. 1 and supplementary table S1) where the haplotypes have been found are added to the haplotype label. Drawing of *Vipera ursinii rakosiensis* courtesy of Márton Zsoldos.

Table 1. Genetic diversity based on 4 microsatellites markers calculated by FSTAT (Goudet, 1995); Ar was evaluated based on 4 diploid individuals.

Subspecies	Location	NB samples	Allelic richness	H_O	H_E	F_{IS}
V. u. rakosiensis	Hanság	23	3.682	0.652	0.675	0.058
	Kiskunság	57	4.200	0.651	0.754	0.146
	Transylvania	40	3.468	0.593	0.658	0.112
	Average		*3.783*		*0.696*	
V. u. moldavica	Iasi	17	3.709	0.238	0.702	0.687
	Danube Delta	44	3.352	0.502	0.652	0.251
	Average		*3.531*		*0.677*	
V. u. macrops	Western Dinarides	56	1.628	0.157	0.206	0.245
	Eastern Dinarides	42	2.894	0.265	0.519	0.499
	Average		*2.261*		*0.363*	
V. u. macrops	Bistra Mt.	6	4.875	0.833	0.760	−0.005
	Average		*4.875*		*0.760*	
V. renardi	Black Sea	30	3.907	0.397	0.625	0.382
	Crimean Ridge	7	2.296	0.176	0.312	0.528
	East European Plains	13	3.578	0.378	0.512	0.301
	Average		*3.260*		*0.483*	
Average all			3.417	0.440	0.580	0.291

The network analyses conducted with PopArt presented a similar structure as the ML, MP and Bayesian inferences (supplementary fig. S1). The position of the samples from Bistra Mt. are confirmed, as well as the strong differentiation between *V. u. macrops* in the southeastern and northwestern part of its distribution range. However, southeastern *V. u macrops* grouped with *V. u. ursinii* from France and Italy as well as *V. graeca*.

nDNA: all regions

All 9 markers were amplified only for *V. u. rakosiensis* (Hanság, Kiskunság and Transylvania regions) and for *V. u. macrops* (Croatia, Montenegro and Bistra Mt.). Only five markers (Vb-D17, Vb-3, Vb-37, Vb-64, Vb-71) were amplified for *V. u. moldavica* and *V. renardi*. Consequently, global analyses were conducted with 5 markers, whereas analyses specifically conducted on *V. u. rakosiensis* and *V. u. macrops* were conducted with 9 microsatellite markers.

Linkage disequilibrium was detected for a couple of primers in two populations only (Kiskunság, Hungary, *V. u. rakosiensis* and Velebit Mt. Croatia, *V. u. macrops*). As it was not the case for all other populations, we decided to keep them for the analyses. For all regions, the genetic diversity was estimated with 5 different markers and the lowest values were recorded for *V. u. macrops* in Croatia (Ar = 2.347 compared to an average value of 3.885) and also in *V. renardi* in the Crimean Ridge (Ar = 2.914; table 1). Similar low levels of H_O and H_E were found in the same populations. The highest values were detected in *V. u. rakosiensis* in Kiskunság (Ar = 4.555), in *V. renardi* in the region of the Black sea (Ar = 4.470) (note that this group contains samples covering a large area), as well as for the 6 samples from Bistra Mt. (localities Medenica and Tonivoda) (Ar = 5.012; *V. u. macrops*). The differences were not significant for Ar ($ANOVA_{4,50} = 1.90$; $p = 0.125$), but were significant for H_O ($ANOVA_{4,50} = 6.678$; $p = 0.0002$) and for H_E ($ANOVA_{4,50} = 2.65$; $p = 0.044$).

Phylogenetic reconstructions separated the different populations of *V. renardi*, *V. u. macrops*, *V. u. rakosiensis* and *V. u. moldavica* (fig. 3). The populations from Bistra Mt. (Medenica and Tonivoda) presented a central position, suggesting a limited relationship with

V. u. macrops. PCoA demonstrated a very close relationship between *V. u. rakosiensis* from Hanság, Kiskunság and Transylvania. Similarly, *V. renardi* populations are very close together, whereas the genetic differentiation is much larger between *V. u. macrops* from Montenegro and from Croatia, or within *V. u. moldavica* (Danube Delta and Iasi). The populations of Bistra Mt. (Medenica and Tonivoda) demonstrated affinities with *V. u. rakosiensis* and *V. u. moldavica*, more than with *V. u. macrops* (supplementary fig. S2a). The PCoA conducted at the individual level again demonstrated the tendencies of gathering individuals by populations and regions, also suggesting similarities between *V. renardi* and *V. u. moldavica*, which was not the case at the population level (supplementary fig. S2b).

Comparison between nDNA and mtDNA

The phylogenetic reconstruction conducted with the microsatellite markers is largely incongruent when compared to mtDNA reconstruction. Indeed, the relative relationships between the different species or subspecies are strongly different between both genomes (fig. 4). Whereas *V. renardi* is strongly differentiated with mtDNA, nuclear genetic distances suggested some affinities between *V. renardi* and *V. u. rakosiensis* even if the bootstrap support is low (49). Whereas *V. u. rakosiensis* and *V. u. moldavica* are sister subspecies according to mtDNA, they are more distant with the nDNA. Moreover, based on the mtDNA, *V. u. macrops* from Western Dinarides showed strong affinities to *V. u. ursinii*, whereas *V. u. macrops* from Eastern Dinarides and Hellenides are more related to the clade formed by *V. u. rakosiensis* + *V. u. moldavica* (see also Ferchaud et al., 2012); on the contrary, nuclear data suggest affinities within northwestern and southeastern populations of *V. u. macrops*. Finally, the position of the samples from Bistra Mt. (Medenica and Tonivoda) is confirmed by both genomes, but the position is strongly different (close to *V. u. moldavica* for the mtDNA, but without specific affinities to one region for the nDNA). Generally speaking, inferred relationships are similar between both nDNA and mtDNA only within subspecies.

Genetic diversity within V. ursinii *subspecies*

nDNA: within V. u. rakosiensis. The subdataset of *V. u. rakosiensis* is composed of 137 samples gathered in 7 populations (3 regions) with 9 microsatellite markers. HW disequilibrium was detected in Hanság for 3 microsatellite markers, whereas disequilibrium was only rarely present in other populations. The genetic diversity is somehow similar between the different regions and different populations (Ar between 3.07 and 4.24, measured on 8 microsatellite markers – Vb-A11 was not amplified for Transylvania 1 and Transylvania 2 – and 4 diploid samples). Population differentiation was limited (F_{ST} varies between 0.04 and 0.14) and significant except for several comparisons including Transylvania 3 (with Transylvania 1, Kiskunság 2 and 3) and Transylvania 1 and Kiskunság 3. Genetic differentiation between populations seems to be unstructured (see supplementary fig. S3a), as the genetic distance is more or less similar between all *V. u. rakosiensis* populations and no bootstrap support higher than 45 was detected. Similarly, the STRUCTURE analysis does not show any tendencies for the best K values (K = 2 following Evanno et al., 2005) but some grouping could be observed for the best L(K) value (K = 5, more or less separating Transylvania, Hanság, Kiskunság 1 and Kiskunság 2 + 3; supplementary fig. S4a). Within *V. u. rakosiensis*, no IBD was detected (Mantel test: $r^2 = 0.070$; $p = 0.247$; supplementary fig. S5).

nDNA: within V. u. moldavica. The subdataset of *V. u. moldavica* is composed of 61 samples gathered in 4 populations (2 regions) with 5 microsatellite markers. The microsatellite markers Vb-D17 was not in HWE in all four populations, and was consequently

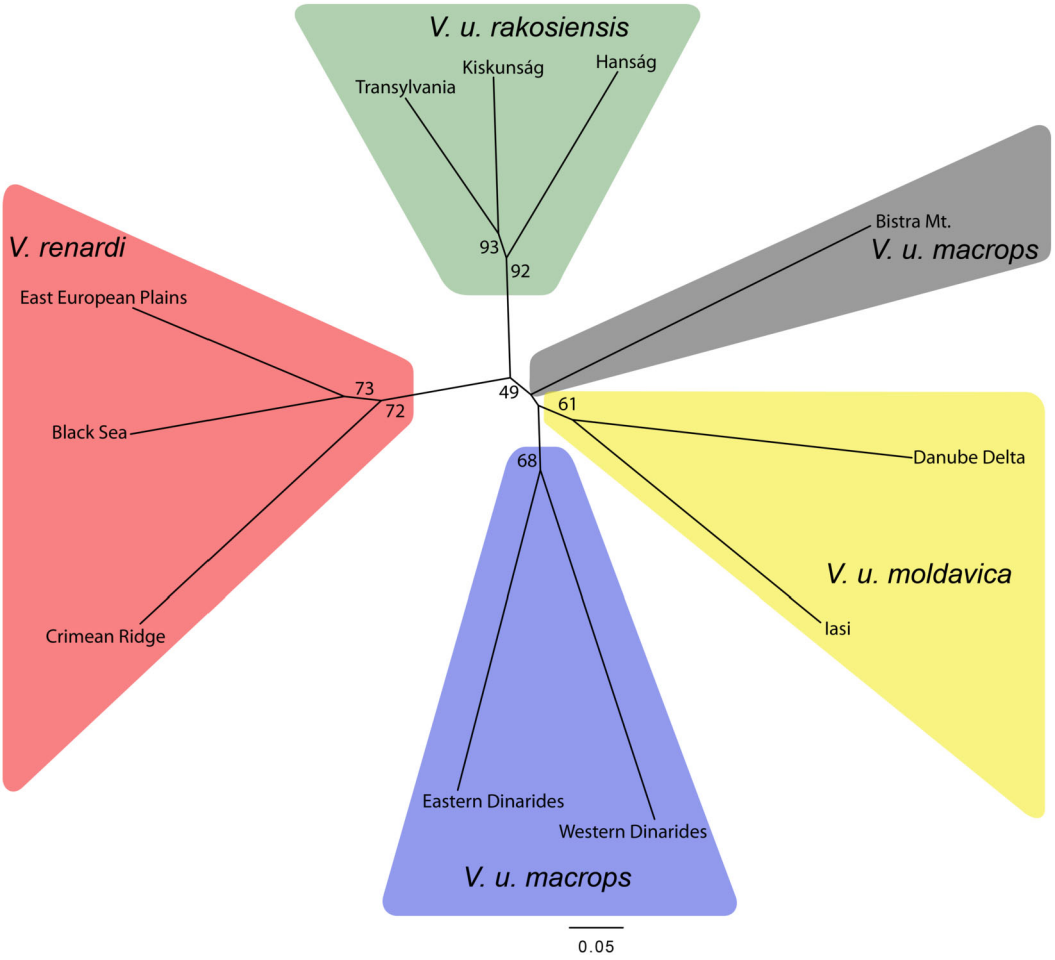

Figure 3. Genetic relationship between locations calculated using Cavalli-Sforza and Edwards *Dc* distance (Cavalli-Sforza and Edwards, 1967) using the software POPULATIONS 1.2.28 (Langella, 1999). The distances were calculated with 5 microsatellite markers and branches with bootstrap support >40 were indicated.

removed from the analyses. The genetic diversity between the different populations was more or less similar (Ar between 4.00 and 5.25, measured on 4 microsatellite markers and 6 diploids samples). Moreover, the genetic divergence is limited between both populations from the Danube Delta (F_{ST} = 0.047), whereas the difference between both populations from Iasi is marked (F_{ST} = 0.177) and even more marked between populations from Iasi and the Danube Delta (F_{ST} between 0.205 and 0.230). All F_{ST} values are significant. Phylogenetic reconstruction highly distinguished both regions (bootstrap support = 100; supplementary fig. S3b), which is confirmed by the Structure analysis (K = 2 for Evanno method; supplementary fig. S4b), as all individuals were largely or completely assigned to one or the other clade according to the region. Within *V. u. moldavica*, no IBD was detected (Mantel test: r^2 = 0.350; p = 0.216; supplementary fig. S5).

nDNA: within V. u. macrops. The subdataset of *V. u. macrops* is composed of 111 samples gathered in 3 regions, with a large sampling (N = 62) in Croatia and in Montenegro (N = 43), and much smaller (N = 6) in Bistra Mt. (localities Tonivoda and Medenica).

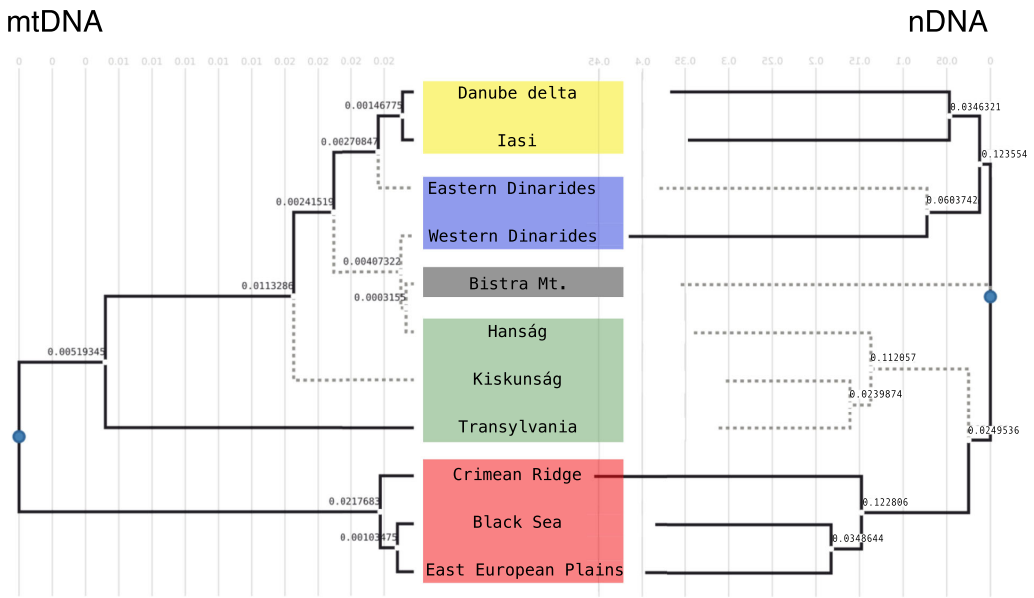

Figure 4. Comparative phylogenetic relationship between the 11 regions with both mtDNA (left) and nDNA (right). left: Mitochondrial DNA tree based on the genetic distances of the different haplotypes (combining cytochrome *b* and ND4; 1920 bp) within each region. right: Nuclear tree based on Cavalli-Sforza and Edwards *Dc* distances (Cavalli-Sforza and Edwards, 1967) calculated with the software POPULATIONS 1.2.28 (Langella, 1999) based on 5 microsatellites markers. Dashed branches correspond to discrepancies between both phylogenetic reconstructions. Both trees were not rooted. The colours are different between subspecies: green: *V. ursinii rakosiensis*, yellow: *V. u. moldavica*, blue: *V. u. macrops*, grey: *V. u. macrops* from Bistra Mt., red: *V. renardi*.

Nine microsatellites were amplified for this subspecies. Large F_{IS} values (F_{IS} = 0.249 and 0.303 for Croatia and Montenegro respectively) in these 2 regions are due to the gathering of individuals from several small populations. Even with this fact, the genetic diversity is very limited in Croatia (Ar = 2.94 measured on 4 microsatellite markers and 6 diploid samples), median for Montenegro (Ar = 4.34), whereas the value is the highest for the Bistra Mt. (locations Medenica and Tonivoda) (Ar = 5.33). Genetic differentiation is strong between Croatia and the two other populations (F_{ST} = 0.354 and 0.404), whereas the differentiation between Montenegro and Bistra Mt. (Medenica and Tonivoda) is lower (F_{ST} = 0.204), all F_{ST} being significant. The Structure analysis separated most individuals of Croatia from samples of Montenegro (K = 2 following Evanno), whereas, with K = 7 (best LN value) distinguished individuals from the three regions, and suggested 2 clusters within Croatia (supplementary fig. S4c). Within *V. u. moldavica*, significant IBD was detected (Mantel test: $r^2 = 0.999$; p = 0.007; supplementary fig. S5).

nDNA between all subspecies. The comparison of IBD conducted separately for each subspecies (also *V. renardi*; $r^2 = 0.093$; p = 0.802; supplementary fig. S5), highlighted the high genetic differentiation observed in the Croatian population. For the other subspecies, a trend to have a higher genetic differentiation within *V. u. moldavica* can be observed (especially compared to *V. u. rakosiensis* or even *V. renardi*). Surprisingly, more intact and continuous populations of *V. renardi* did not show much higher level of genetic diversity, probably due to recent expansion of this species to East European grasslands. The highest genetic diversity in *V. renardi* (Ar = 3.907) was registered in the pooled Black sea region sample, however

the contact zone between two mitochondrial lineages there may explain it and high F_{ST} values are supporting this hypothesis.

Discussion

mtDNA-nDNA similarities and cytonuclear discordance

Within subspecies, mtDNA and microsatellite markers globally showed similarities in their genetic structure. However, the structure between the subspecies is not really resolved with the nDNA analysis conducted here, probably due to the high variability within microsatellite markers. Except for the position of the individuals from Bistra Mt. (Tonivoda and Medenica localities, see discussion below), the mtDNA analyses are congruent with what had already been found and published in Gvozdik et al. (2012), Ferchaud et al. (2012), Zinenko et al. (2015), or more recent publications related to these species. The analyses of nDNA, here based on several microsatellites markers, and also on three nuclear sequence markers in Mizsei et al. (2017), demonstrated that both parts of the genomes have a similar signal. Congruence between nDNA and mtDNA suggests that the observed signal represents the real phylogenetic history of this species. A recent introgression between the different groups/subspecies is consequently unlikely or has not been conducted to genetic introgression to a high level. In combination with the genetic analyses, the reconstruction of the putative past distribution range of the different genetic clusters using past and current climatic variables would help to understand the movement of the species during the last Pleistocene.

The polyphyletic position of *V. u. mac*rops based on mtDNA observed in this study and in previous ones (e.g., Ferchaud et al., 2012) is not supported by the nDNA analyses. Indeed, whereas northwestern populations (Western Dinarides) of *V. u. macrops* are more related to *V. u. ursinii*, the southeastern populations (Eastern Dinarides and Hellenides) are linked to the clade formed by *V. u. rakosiensis* and *V. u. moldavica*, based on mtDNA analyses. On the opposite, microsatellite markers suggested that Eastern and Western Dinarides populations of *V. u. macrops* are related, without any connection to *V. u. rakosiensis* or *V. u. moldavica*. A closer look at the nDNA markers in Mizsei et al. (2017), especially PRLR, also suggested a similar pattern, with identical alleles in all regions of *V. u. macrops* and small differences with other subspecies (*V. u. rakosiensis* or *V. u. moldavica*, but also *V. u. ursinii*). We can thus hypothesise that, even if *V. u. macrops* is paraphyletic for the mtDNA, past gene exchanges in the mountains of the Balkan Peninsula were sufficient to have maintained similarities in nuclear genes. However, these conclusions should be considered with caution as only a limited number of nuclear loci have been analysed. Confronting mtDNA phylogenies with phylogenetic analyses based on numerous nuclear loci (e.g., with SNPs) would probably help to better understand the relationship within *V. u. macrops* populations and determine if two subspecies should be considered.

Genetic diversity within V. ursinii

The populations of *V. u. rakosiensis* and *V. u. moldavica* are known to be the smallest and most threatened ones within *V. ursinii* (Nilson and Andrén, 2001; Edgar and Bird, 2005; Halpern, 2007). The use of highly polymorphic markers like microsatellites allowed us to evaluate the genetic diversity between most *V. ursinii* subspecies, and even compare it to geographically close populations of *V. renardi*. However, the values observed in both *V. u. rakosiensis* and *V. u. moldavica* are higher than in *V. u. macrops* and at a similar level compared to *V. renardi*. Consequently, this observation suggests that, even if the populations are currently small, isolated and fragmented, they still have retained a large and probably sufficient genetic diversity, suggesting a very recent population size reduction.

Interestingly, the lowest genetic diversity was found in Western Dinarides (*V. u. macrops*), in the Crimean ridge (*V. renardi*), and, to a lower extent, in the Montenegrin *V. u. macrops* population (table 1). While low values for those regions could be related to a limited sampling area, the mountain populations of *V. u. macrops* are large, and probably interconnected (or were interconnected in the past). This low genetic diversity is perhaps resulting from very recent bottlenecks, or from past ones happening during the Last Glacial Maximum (LGM), or even during interglacial periods, when the populations had to go lower or higher in altitude in the mountains. Indeed, it has already been demonstrated that low genetic diversity evaluated with microsatellite markers could be a sign of post LGM recolonisation (Ursenbacher et al., 2015), and that populations at a long distance to glacial refugia have lower genetic diversity.

On the opposite, the highest value (Ar and H_E) was detected in the population from Bistra Mt. (localities Medenica and Tonivoda), where two different groups of mtDNA (similar to *V. u. moldavica* and as a sister group of *V. u. rakosiensis*) were identified. This high genetic diversity both in mtDNA and nDNA could be related to a newly formed population from several origins of previously divergent populations, be an old refugial area or a centre of speciation for *V. ursinii* (see below).

Individuals from Bistra Mt.: historical hypothesis

Both mtDNA and nDNA suggest a particular history in the population from Bistra Mt., even if these two genome types of different heritability do not suggest the same historical reconstruction. Samples from these two locations (separated by 4 km) are geographically very close to other populations of *V. u. macrops*, whereas mtDNA data suggested affinities to *V. u. rakosiensis* and *V. u. moldavica* (fig. 2). Moreover, the nuclear genome of this population does not show affinity with other geographically closer *V. u. macrops* populations. Based on the geographical location of Bistra Mt. and its occurrence in the southern part of the distribution range of *V. u. macrops*, the affinites to lowland subspecies were not expected. The possibility of a recent human-mediated introduction from a current population is unlikely as 1) the location is highly isolated; 2) some mtDNA haplotypes are very different from all known sampled populations; 3) the nDNA signal is also completely different from all analysed populations, and 4) the genetic diversity is not reduced as expected in the case of an introduction. Consequently these two locations (likely forming a single population) are probably resulting respectively from an old colonisation event from lowland subspecies, be the centre of diversification within *V. ursinii*, or be a remnant of extinct populations located more east and related to lowland subspecies. Our results are, however, based on a limited number of samples (N = 6), and thus additional sampling in this area, as well as surrounding populations, is necessary to 1) determine the limit of this genetic cluster; 2) identify its genetic diversity, thus 3) provide more stable hypotheses on the phylogenetic position, and 4) propose a reconstruction of past history of this cluster which could completely change the historical reconstruction of *V. ursinii*.

Vipera u. rakosiensis – V. u. moldavica

For *V. u. moldavica*, two regions (Iasi and Danube Delta), each comprising 2 separate sites, were analysed. The genetic differentiation within this group is marked, except between both populations from the Danube Delta. The analyses conducted with Structure also suggested that these two regions are largely genetically separated. Nevertheless, the within-region level of divergence is somewhat surprising, as the two sites from the Danube Delta are separated by a greater distance (30-40 km) but a low genetic differentiation ($F_{ST} = 0.047$) compared to the two sites from the Iasi region (4.5-7.5 km; $F_{ST} = 0.177$). The large discrepancy can however be explained by the quality of the habitat

between populations. Indeed, the two Iaşi sites are separated by anthropically modified landscapes, such as agricultural fields, roads, and human settlements, while there are only natural barriers (most importantly the Sfântu Gheorghe branch of the Danube river) separating the two deltaic populations.

On the opposite, genetic differentiation within *V. u. rakosiensis* populations is lower and weakly explained by the geographic distance between the populations ($r = 0.264$). These results are typical for large, continuous populations, which is currently not the case. Until the second half of the 20th century typical steppe habitats inhabited by *V. u. rakosiensis* were much more extended (Péchy et al., 2015). Moreover, the main rivers (Danube and Tisza; see fig. 1) and their tributaries encompassing the Carpathian Basin were strongly regulated in the 19th century causing significant change in the water level of the Basin (Chu, 2018), affecting the structure of the viper habitats as well. As the average genetic diversity is still high and quite similar between all tested regions of *V. u. rakosienis* and *V. u. moldavica* (table 1), it is very likely that population sizes were large until the last century, and loss and fragmentation of natural range restricted the once large populations into tiny fractions of their distribution area. As this species can live in high densities (Cheylan et al., 2011), the effective population size could locally stay quite large until the last decades. Moreover, due to the high generation time in snakes (about 5-8 years; see Baron et al., 1996), most of the genetic diversity was retained until now. The genetic markers used are not able to show the recent structure and population size reduction – thus they are still retaining and displaying the genetic structure and differentiation that was present a few decades/centuries ago. Some differences in genetic diversity between regions in *V. u. rakosiensis* have been observed – populations from Hanság, Kiskunság 1, Kiskunság 2 + 3, and Transylvania are separated by Structure (supplementary fig. S4-a2) – probably reflecting the ongoing isolation process. The most stunning discovery is the relatively high genetic diversity of the largest population from Kiskunság (supplementary table S3), contradicting previous findings. Indeed, Ujvári et al. (2002) studied the genetic variability at the major histocompatibility (Mhc) class I loci in *V. u. rakosiensis*, analysing 8 individuals originating from 4 different localities of Kiskunság region. They concluded that genetic diversity is extremely low in both some of the northern (Dög-hegy (Kiskunság 3 in our study)) and the southern (Bugac (Kiskunság 1 in our study)) localities, however they found distinct haplotypes in two other northern localities (Dabas – Kiskunság 2 and Peszér – Kiskunság 3 in our study), based on single individuals. Even though our findings are partially conflicting, they involved different sampling sizes as well as marker types (microsatellites are mostly not under selection while strong selection occurs on MHC genes) which may explain the different results.

General conclusion

The combination of both mtDNA and nDNA genomes demonstrated congruences for most *V. ursinii* subspecies. Only *V. u. macrops* (including the population of Bistra Mt.) seems to harbour several mtDNA lineages with strongly different affinities which is not underlined by the nDNA history. The use of next generation sequencing with the study of a large portion of the genome, in combination with a better sampling, especially in the southern part of its distribution, would possibly help to resolve the incomplete resolution of this study. Globally, *V. ursinii*, and more specifically *V. u. rakosiensis* are considered as one of the most threatened snake species in Europe. The genetic markers used here, however, do not display strong genetic reduction in Southeastern, Eastern and Central Europe, probably reflecting past history of the taxa. Nevertheless, the only way to maintain this genetic diversity and to avoid

an increase in genetic differentiation between populations in the future is mainly to recreate suitable habitats in the surroundings of current populations (as conducted by several ongoing LIFE projects) and when possible, reconnect them.

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