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Nuclear markers support the mitochondrial phylogeny of *Vipera ursinii–renardi* complex (Squamata: Viperidae) and species status for the Greek meadow viper

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Abstract

Meadow vipers (*Vipera ursinii–renardi* complex) are small-bodied snakes that live in either lowland grasslands or montane subalpine-alpine meadows spanning a distribution from France to western China. This complex has previously been the focus of several taxonomic studies which were based mainly on morphological, allozyme or immunological characters and did not clearly resolve the relationships between the various taxa. Recent mitochondrial DNA analyses found unexpected relationships within the complex which had taxonomical consequences for the detected lineages. The most surprising was the basal phylogenetic position of *Vipera ursinii graeca*, a taxon described almost 30 years ago from the mountains of Greece. We present here new analyses of three nuclear markers (*BDNF*, *NT3*, *PRLR*; a first for studies of meadow and steppe vipers) as well as analyses of newly obtained mitochondrial DNA sequences (*CYT B*, *ND4*).Our Bayesian analyses of nuclear sequences are concordant with previous studies of mitochondrial DNA, in that the phylogenetic position of the *graeca* clade is a clearly distinguished and distinct lineage separated from all other taxa in the complex. These phylogenetic results are also supported by a distinct morphology, ecology and isolated distribution of this unique taxon. Based on several data sets and an integrative species concept we recommend to elevate this taxon to species level: *Vipera graeca* Nilson & Andrén, 1988 **stat. nov.**

Key words: Albania, Balkan Peninsula, endemic, Greece, nDNA, Pindos mountains, snake, subspecies

Introduction

Meadow vipers (*Vipera ursinii–renardi* complex) are small-bodied vipers that live in either lowland grasslands or montane subalpine-alpine meadows. Most of these taxa have highly fragmented distributions, spanning from eastern France to western China (Nilson & Andrén 2001). European members of the *V. ursinii–renardi* complex are among the most endangered species of the European herpetofauna and their systematics and evolutionary histories have been in the focus recently (see Nilson & Andrén 2001 for review and Tuniyev *et al.* 2010; Ferchaud *et al*. 2012; Gvoždík *et al*. 2012; Zinenko *et al.* 2015). Based on an extensive analysis of morphology, three groups have been detected within the *V. ursinii–renardi* complex (Nilson & Andrén 2001); the *ursinii* group ("meadow vipers": *ursinii*, *macrops*, *graeca*, *moldavica*, *rakosiensis*), the "Transcaucasian–Turkish" group (*anatolica*, *ebneri*, *eriwanensis*, *lotievi*), and the *renardi* group ("steppe vipers": *renardi*, *altaica*, *tienshanica*, *parursinii*). Over the past two decades, some of the formerly recognized subspecies within these groups were elevated to full species

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status based on morphological, allozyme or immunological differences (e.g. *V. eriwanensis* Nilson *et al.* 1993, *V. renardi* Kotenko *et al*. 1999; Nilson & Andrén 2001, *V. anatolica* and *V. ebneri* Nilson & Andrén 2001).

The molecular phylogeny of meadow and steppe vipers has not been fully resolved due to limited knowledge on certain taxa or inadequate sampling in analyses, although great strides have been made and clarified several evolutionary relationships (Kalyabina-Hauf *et al.* 2004; Ferchaud *et al*. 2012; Gvoždík *et al*. 2012; Zinenko *et al.* 2015). According to the latest mitochondrial phylogeny, the most recent common ancestor of the complex has Pliocene origin, located presumably in the region between the eastern Mediterranean and the former Paratethys (Zinenko *et al.* 2015). Members of the major mitochondrial clades probably radiated in the Anatolian-Transcaucasian region (*renardi* clade) and on the Balkan Peninsula (*ursinii* clade) with subsequent colonization patterns to the western and eastern parts of central Europe forming the present geographic distributions (Ferchaud *et al*. 2012; Zinenko *et al.* 2015).

Based on these most recent studies, there is a need for taxonomical investigation and reassessments for taxa with unexpected phylogenetic positions in the whole genus; or more specifically the *V. ursinii–renardi* complex (see Ferchaud *et al.* 2012; Zinenko *et al.* 2015). In such a case, nuclear DNA loci (nDNA) could help corroborate the pattern and be used to better resolve the evolutionary relationships and taxonomy of meadow vipers, but this has yet to be done.

A member of the genus with a surprising phylogenetic placement is the Greek meadow viper, *Vipera ursinii graeca* Nilson & Andrén, 1988, which is a rare taxon described three decades ago. The species lives in high elevation meadows (1600–2300 m) of the central and southwestern Hellenides mountain range (Dimitropoulos 1985; Nilson & Andrén 1988; Korsós *et al.* 2008; Mizsei *et al.* 2016). According to the phylogenetic study of Ferchaud *et al.* (2012), *V. u. graeca* is sister to all other clades of *ursinii* and *renardi* in the *V. ursinii–renardi* complex and forms a distinct, divergent clade sister to the others, isolated in fragmented habitats in the Pindos mountains. Similar results have been reported by Zinenko *et al.* (2015) who also included samples from *Vipera anatolica*, and a seemingly relict population of *Vipera kaznakovi* which is highly divergent. As there is fairly strong consensus between taxonomic units in reptiles using mitochondrial DNA (mtDNA) analyses (Joger *et al*. 2007), Ferchaud *et al.* (2012) proposed *V. u. graeca* as a possible candidate for full species status. However, taking a multilocus approach, in this day and age, is the most widely used and strongly supported data and results when discerning species and implementing taxonomic assessments (Torstrom *et al.* 2014). Using molecular methods, taxonomy is currently experiencing a new boom with many re-evaluated or newly described reptile taxa also in such well-known region as is Europe (e.g. *Dinarolacerta*, *Malpolon*; Carranza *et al.* 2006; Ljubisavljević *et al*. 2007 and Speybroeck *et al*. 2010 for review). Therefore, the aim of our study was to analyse nDNA loci in the *V. ursinii-renardi* complex, to see if the results concur with previous mtDNA studies, and resolve the taxonomic status and phylogenetic placement of *V. u. graeca*.

Material and methods

Geographic and taxon sampling. Two samples of *V. u. graeca* from Albania were included in this study (Mizsei *et al.* 2016), both representing the same mtDNA haplotype as the published sequences from Greece in previous studies (*CYT B*, *ND4*; Ferchaud *et al.* 2012). Other specimens investigated were samples representing montane populations of *Vipera ursinii macrops* (Albania, Montenegro), *V. u. ursinii* (Italy), *V. ursinii* ssp. (an undescribed lineage from Croatia), lowland vipers *V. u. moldavica* (Romania) and *V. u. rakosiensis* (Hungary, Romania), *V. renardi* (Crimea) as well as three subspecies of the common adder *V. berus*; *berus* (Hungary), *bosniensis* (Albania) and *nikolskii* (Romania) (see Fig. 1 and Table 1 for details) as outgroup taxa.

DNA extraction and sequencing. We selected two mitochondrial markers (*CYT B*, *ND4*) and three nuclear markers (*BDNF*, *NT3*, *PRLR*) shown to be successful in discriminating on various divergence levels among several reptile species (Joger *et al*. 2007; Townsend *et al*. 2008). We used the DNeasy Blood & Tissue Kit (Qiagen) and the NucleoSpin Tissue kit (Macherey-Nagel) for extracting total genomic DNA. Polymerase chain reaction (PCR) conditions for amplifying mitochondrial *CYT B* and *ND4* genes were followed protocols outlined in Ferchaud *et al.* (2012). The genes were amplified using the primers shown in Table 2. PCR conditions for *PRLR* and *BDNF* were as follows; 180 seconds at 94°C, followed by 40 steps of 94°C (40s), 50°C (30s), 72°C (60s) and a final elongation step of 7 min at 72°C. PCR conditions for *NT3* were 180 seconds at 94°C, followed by 40 steps of 94°C (40s), 48°C (30s), 72°C (60s) and a final elongation step of 7 min at 72°C.

TABLE 1. List of samples used in this study and GenBank accession numbers of sequenced markers.

Taxon	ID in this study	Country	Population	mitochondrial DNA		nuclear DNA		
				CYT B	***ND4***	***PRLR***	***BDNF***	***NT3***
Vipera berus berus	Vbbe-HU	Hungary	Zemplén	JN204721 † **	LT220998 ●	LT221007 ●	LT220962 ●	LT220980 ●
Vipera berus bosniensis	Vbbo-AL	Albania	Prokletije	LT220959 ●	LT220999 ●	LT221008 ●	LT220963 ●	LT220981 ●
Vipera berus nikolskii	Vbni-RO	Romania	Iaşi	-	LT221005 ●	LT221018 ●	LT220973 ●	LT220991 ●
Vipera ursinii ursinii	Vuur-IT	Italy	Camerino	FR727041 ‡ *	LT221006 ●	LT221024 ●	LT220979 ●	LT220997 ●
Vipera ursinii ssp.	Vurs-HR1	Croatia	Velebit	FR727052 ‡	FR726984 ‡	LT221009 ●	LT220964 ●	LT220982 ●
Vipera ursinii ssp.	Vurs-HR2	Croatia	Velebit	FR727053 ‡	FR726985 ‡	LT221010 ●	LT220965 ●	LT220983 ●
Vipera ursinii macrops	Vumc-AL1	Albania	Korab	LT220961 ●	LT221000 ●	LT221013 ●	LT220968 ●	LT220986 ●
Vipera ursinii macrops	Vumc-MN1	Montenegro	Bjelasica	FR727058 ‡ *	LT221001 ●	LT221014 ●	LT220969 ●	LT220987 ●
Vipera ursinii macrops	Vumc-MN2	Montenegro	Bjesalica	FR727059 ‡ *	LT221002 ●	LT221015 ●	LT220970 ●	LT220988 ●
Vipera ursinii rakosiensis	Vura-HU1	Hungary	Nagypuszta	FR745956 ‡	FR745905 ‡	LT221019 ●	LT220974 ●	LT220992●
Vipera ursinii rakosiensis	Vura-RO	Romania	Csengerpuszta	FR745989 ‡	FR745901 ‡	LT221020 ●	LT220975 ●	LT220993 ●
Vipera ursinii rakosiensis	Vura-HU2	Hungary	Fűzfa-szigetek	FR745959 ‡	FR745900 ‡ *	LT221021 ●	LT220976 ●	LT220994 ●
Vipera ursinii moldavica	Vuml-RO1	Romania	Iaşi	JN204699 † *	LT221003 ●	LT221016 ●	LT220971 ●	LT220989 ●
Vipera ursinii moldavica	Vuml-RO2	Romania	Iaşi	JN204700 † *	LT221004 ●	LT221017 ●	LT220972 ●	LT220990 ●
Vipera renardi	Vren-CM1	Ukraine	Crimea	FR745991 ‡ *	FR745893 ‡ *	LT221022 ●	LT220977 ●	LT220995 ●
Vipera renardi	Vren-CM2	Ukraine	Crimea	FR745992 ‡ *	FR745894 ‡ *	LT221023 ●	LT220978 ●	LT220996 ●
Vipera graeca **stat. nov.**	Vgre-AL1	Albania	Dhëmbel	LT220960 ●	LN835177 ♦	LT221011 ●	LT220966 ●	LT220984 ●
Vipera graeca **stat. nov.**	Vgre-AL2	Albania	Trebeshinë	HG940677 ● *	HG940672 ♦	LT221012 ●	LT220967 ●	LT220985 ●

†Gvoždík *et al.* 2012; ‡Ferchaud *et al.* 2012; ♦Mizsei *et al.* 2016; ●present study;

* not the same individual as for nDNA sequencing, but same population;

** not the same individual as for nDNA sequencing, but same taxon; - missing sequence.

TABLE 2. Primers used in this study.

Primer	Gene	Reference	Sequence
L14724Vb	*CYT B*	Ursenbacher *et al.* 2006	5'-GATCTGAAAAACCACCGTTG-3'
H15914Vb	*CYT B*	Ursenbacher *et al.* 2006	5'-AAATAGAAAGTATCATTCTGGTTTAAT-3'
ND4	*ND4*	Arevalo *et al.* 1994	5'-CACCTATGACTACCAAAAGCTCATGTAGAAGC-3'
H12763V	*ND4*	Wüster *et al.* 2008	5'-TTCTATCACTTGGATTTGCACCA-3'
BDNFf	*BDNF*	Townsend *et al.* 2008	5'-GACCATCCTTTTCCTKACTATGGTTATTTCATACTT-3'
BDNFr	*BDNF*	Townsend *et al.* 2008	5'-CTATCTTCCCCTTTTAATGGTCAGTGTACAAAC-3'
NT3-F3	*NT3*	Noonan & Chippindale 2006	5'-ATATTTCTGGCTTTTCTCTGTGGC-3'
NT3-R4	*NT3*	Noonan & Chippindale 2006	5'-GCGTTTCATAAAAATATTGTTTGACC-3'
PRLR_f1	*PRLR*	Townsend *et al.* 2008	5'-GACARYGARGACCAGCAACTRATGCC-3'
PRLR_r3	*PRLR*	Townsend *et al.* 2008	5'-GACYTTGTGRACTTCYACRTAATCCAT-3'

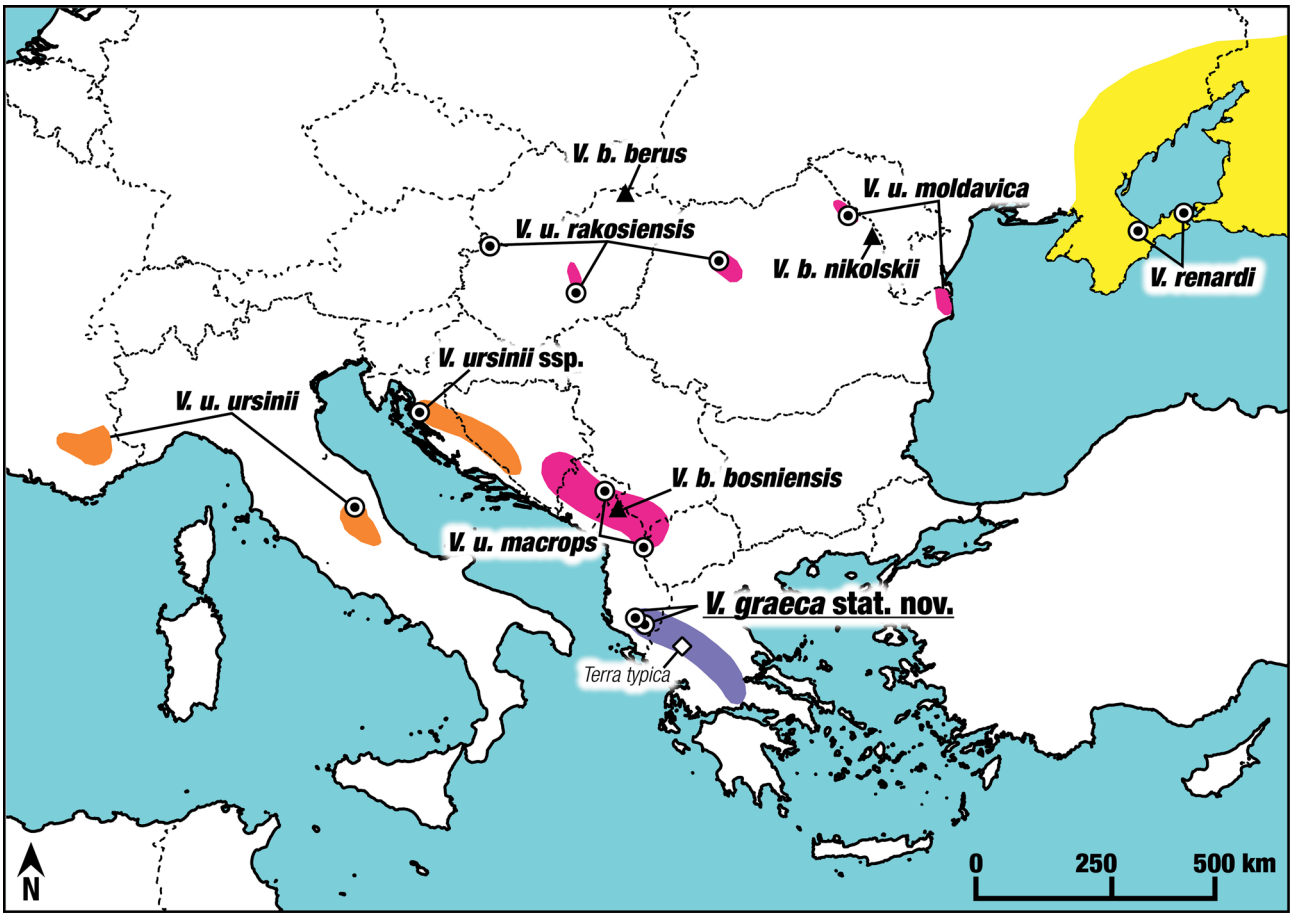

FIGURE 1. Sampled localities inside the approximate distribution area of *Vipera ursinii–renardi* complex in Europe. Circles indicate sampling localities of *Vipera ursinii–renardi* complex, and triangles show the sampling of outgroup taxa. A diamond indicates the type locality of *Vipera graeca* **stat. nov**. This figure is published in colour in the online version, the colour of the patches corresponds to the colour of mitochondrial lineages in Fig. 2A.

PCR products were purified with High Pure PCR Product Purification Kit (Roche) or on NucleoFast 96 PCR plates (Macherey-Nagel) using vacuum filtering. DNA sequencing was performed using BigDye v1.1 (Life Technologies) for cycle sequencing reaction and DNA sequencing was performed on an ABI 3130xl capillary sequencer (Life Technologies).

Phylogenetic analyses. Sequences were assembled and aligned using CodonCode Aligner v5 (CodonCode Corp.) and chromatograms were checked visually in order to clean the sequences. Coding gene fragments were

trimmed and translated into amino acids; no stop codons were observed. For this study, a complete dataset of two mitochondrial and three nuclear markers was analysed for all samples/localities, except the *Vipera berus nikolskii* specimen where *CYT B* sequence was missing and the gap was replaced by "N" in the analysis.

Heterozygous positions in nuclear genes were manually identified based on the presence of double peaks in chromatograms. Identified heterozygous loci were coded according to the IUPAC ambiguity codes.

For the purpose of allele network construction, sequences with more than one heterozygous position (detected in the presence of two peaks of approximately equal height at a single nucleotide site of *BDNF*, *NT3*, *PRLR*) were resolved in PHASE 2.1.1 (Stephens *et al.* 2001) for which the input data for PHASE were prepared in SeqPHASE (Flot 2010). PHASE was run under default settings except the probability threshold, which was set to 0.7. Haplotype networks of the three nuclear markers (*BDNF*, *NT3*, *PRLR*) were drawn using TCS 1.21 (Clement *et al.* 2000) with 95% connection limit. New sequences were deposited in the GenBank (Table 1).

The best-fit codon-partitioning schemes and the best-fit substitution models for phylogenetic analyses were selected using PartitionFinder v1.1.1. (Lanfear *et al*. 2012) separately for each dataset and methodological approach (i.e. models available in the used software) with the following parameters: Bayesian approach (BA) - linked branch length; all models; BIC model selection; greedy schemes search; data blocks by codons for each used marker. The best partitioning scheme and models of nucleotide substitutions were: 1st position of *CYT B* (TrN), 2nd position of *CYT B* (HKY + I), 3rd position of *CYT B* (HKY), 1st + 3rd positions of *ND4* (HKY + I), 2nd position of *ND4* (HKY + G), *BDNF* (K80), *NT3* (K80), *PRLR* (TrN). A Maximum likelihood (ML) analysis following the same procedure as above (the best model in this case was the GTR+G+I with a single partition). The number of variable (*V*) and parsimony informative (*Pi*) sites were calculated in DnaSP 5.10 (Librado & Rozas 2009).

To resolve phylogenetic relationships we analysed the own obtained dataset consisting of mitochondrial (1788 bp) and nuclear (1715 bp) sequence data. To show and confirm relationships based on the mtDNA (full sequences for all taxa of *ursinii–renardi* complex were available only for *CYT B*; 1116 bp) we recalculated dataset of Ferchaud *et al.* (2012) together with a *Vipera anatolica* sequence of Zinenko *et al.* (2015) and our new *graeca* sequences. Mitochondrial phylogenetic trees were inferred using the BA performed with MrBayes 3.2.1 (Ronquist *et al.* 2012) and ML analysis performed with RAxML 8.0 (Stamatakis 2014). Each codon position treated separately was selected as the best-fit partitioning scheme for BA (see above). The BA analysis was set as follows; two separate runs, with four chains for each run, 10 million generations with trees sampled every 100th generation. First 20% of trees were discarded as the burn–in after inspection for stationarity of log–likelihood scores of sampled trees in Tracer 1.6 (Rambaut *et al*. 2013; all parameters had effective sample sizes of > 200). A majority–rule consensus tree was drawn from the post–burn–in samples and posterior probabilities were calculated as the frequency of samples recovering any particular clade. The same procedure was performed with the concatenated dataset of all genes with a final length of 3503 bp. Sequences of nuclear genes were not phased and partitioned by genes due to low level of divergence. Both protein-coding genes (*CYT B*, *ND4*) were partitioned by codon position. Nodes with posterior probability (pp) values ≥ 0.95 were considered as strongly supported. The ML clade support was assessed by 1000 bootstrap pseudoreplicates.

The NeighborNet algorithm (Bryant & Moulton 2004) implemented in the software SplitsTree 4.10 (Huson & Bryant 2006) was used to generate a phylogenetic network of the phased dataset. To assess the support for the observed structure, bootstrap analysis was performed with 1000 pseudoreplicates. Nodes were considered strongly supported if they received bootstrap values > 70%. This phylogenetic analysis is a powerful tool for visualizing conflicting and consistent information present in the dataset (Huson & Bryant 2006).

Species tree estimation. Coalescent-based species tree estimation (STE) was performed with *BEAST v.1.8.0 (Drummond *et al.* 2012a) with the same dataset used in the phylogenetic network analysis. Because *BEAST assumes no recombination within loci (Heled & Drummond 2010), we tested for the presence of recombination within all nuclear loci analysed using RDP4 (Martin *et al.* 2010). Alignments of both mtDNA and all four nDNA genes were imported independently into BEAUti 1.7.5 (Drummond *et al.* 2012a). Nuclear genes were phased prior to analysis as described above. Three individual runs were performed for 5×10^7 generations with a sampling frequency of 5000. Appropriate substitution models are specified as above and priors applied are as follows (otherwise by default): Coalescence–Yule process of speciation; random starting tree, substitution rate fixed to 1; strict clock; base substitution Uniform (0, 100); alpha Uniform (0, 100); initial = 0.5; clock rate Uniform (0, 1). Parameter values both for clock and substitution models were unlinked across partitions and trees for the mtDNA partitions were linked. Each run of STE was analysed in Tracer v.1.6 (Rambaut *et al.* 2013) to confirm that

stationarity, convergence and effective sample sizes (ESS) were sufficient for all parameters (posterior ESS values > 300). LogCombiner and TreeAnnotator (both available in *BEAST package) were used to infer the ultrametric tree after discarding 10% of the samples from each run and the production of the chronogram. A maximum clade credibility tree from the sampled trees was produced using TreeAnnotator. Apart from producing a maximum clade credibility tree of the full dataset (mtDNA + nDNA), we visualized all post burn-in sampled trees from all three runs (27 000 trees) using DensiTree 2.1.11 (Bouckaert 2010), which allows superimposing all the sampled trees. Nodes were considered strongly supported if they received posterior probability (pp) values > 0.95.

Results

Our dataset included 18 specimens of the genus *Vipera*; three samples of *Vipera berus* for outgroup (subspecies *berus*, *bosniensis* and *nikolskii*) as a species phylogenetically very close to the *Vipera ursinii–renardi* complex, two samples of *Vipera renardi* complex and 13 samples of *Vipera ursinii* complex (subspecies *graeca*, *macrops*, *moldavica*, *rakosiensis*, *ursinii* and *ursinii* ssp. from Croatia; see Ferchaud *et al.* 2012, Zinenko *et al.* 2015 for details about mitochondrial phylogeny). The dataset included mitochondrial gene fragments of *CYT B* (1043 bp, *V* = 142, *Pi* = 107) and *ND4* (747 bp, *V* = 101, *Pi* = 80) and nuclear gene fragments of *BDNF* (663 bp, *V* = 5, *Pi* = 4), *NT3* (497 bp, *V* = 14, *Pi* = 10) and *PRLR* (553 bp, *V* = 17, *Pi* = 15) totalling to 3503 bp (Table 1). No evidence of recombination was detected within the nuclear loci.

Phylogenetic reconstructions and allele networks. Phylogenetic analyses (BA, ML) and phylogenetic network (Fig. 2) constructed from the mitochondrial as well as from the concatenated dataset resulted in a topology concordant with main clades as observed in the mtDNA phylogeny of Ferchaud *et al.* (2012) and Zinenko *et al.* (2015) (Fig. 2). High Bayesian posterior probabilities (≥ 0.95; Fig. 2A, 2B) and bootstrap support values (> 70; Fig. 2A,B,C) were noted for the *graeca* clade as well as for most of other included clades (Fig. 2). Concatenated mtDNA + nDNA data set revealed four deeply divergent clades within the *ursinii–renardi* complex, with a high degree of structure corresponding to divergent lineages (see Fig. 2). There is strong support for the *graeca* clade and for clade covering the *V. u. moldavica*, *V. u. rakosiensis* and *V. u. macrops* subclades. Similarly to mtDNA tree, a clade covering *V. u. ursinii* and *V. ursinii* ssp. was not supported by BA analysis. The clade covering *Vipera renardi–eriwanensis* subclades is not supported in concatenated dataset probably due to missing data of *eriwanensis* subclade.

The networks constructed for the phased haplotypes of the full length nuclear markers *BDNF* (6 unique haplotypes), *NT3* (9 haplotypes) and *PRLR* (12 haplotypes) are presented in Fig. 3. A very low level of haplotype variability was detected for the *BDNF* marker. The *NT3* marker was more variable with five haplotypes within the *ursinii* group. The most variable marker was the *PRLR* with six haplotypes in the *ursinii* group. This marker shows Vumc-MN1b allele among two alleles of Croatian population probably due to incomplete lineage sorting (= ancestral polymorphism). Alleles of the *graeca* clade belong to distinct haplotypes in all three nuclear loci, however, they share a haplotype of *BDNF* with Italian *V. u. ursinii*. These results indicate that alleles of *NT3* and *PRLR* are clearly unique for *graeca*, and in case of *NT3,* the haplotype of *graeca* is highly diverged from all other taxa by six mutation steps (Fig. 3). Furthermore, alleles of the *graeca* lineage always represent one particular haplotype which support evolutionary distinction among the taxa analysed including *V. berus*.

Species tree. All three independent *BEAST runs converged, ESS values of all parameters in all runs exceeded 200, a critical value suggested by the *BEAST manual (Drummond *et al*. 2012b) indicating adequate mixing of the MCMC analyses. The ESS of the likelihoods was > 4000. The topology inferred from the maximum clade credibility species tree based on the mitochondrial and nuclear loci was the same as the mtDNA gene tree of Ferchaud *et al.* (2012), and similar to the topology Zinenko *et al.* (2015) (Fig. 2A). The graeca lineage is sister to all other members of the *ursinii–renardi* group, and *V. renardi* is the sister lineage to the *ursinii* group. Within the *V. ursinii* group, the montane vipers of *V. u. ursinii* and *V. ursinii* ssp. from Croatia form a clade sister to a lowland-montane clade of *V. u. rakosiensis*, *V. u. moldavica* and *V. u. macrops*. Most relationships were highly supported (> 0.95) except the relationship between *V. u. ursinii* and *V. ursinii* ssp.. Considering the focus of this study, the *graeca* lineage was confirmed as a highly supported and basal lineage within the *V. ursinii–renardi* complex according to the *BEAST analysis (Fig. 4, pp = 1.00).

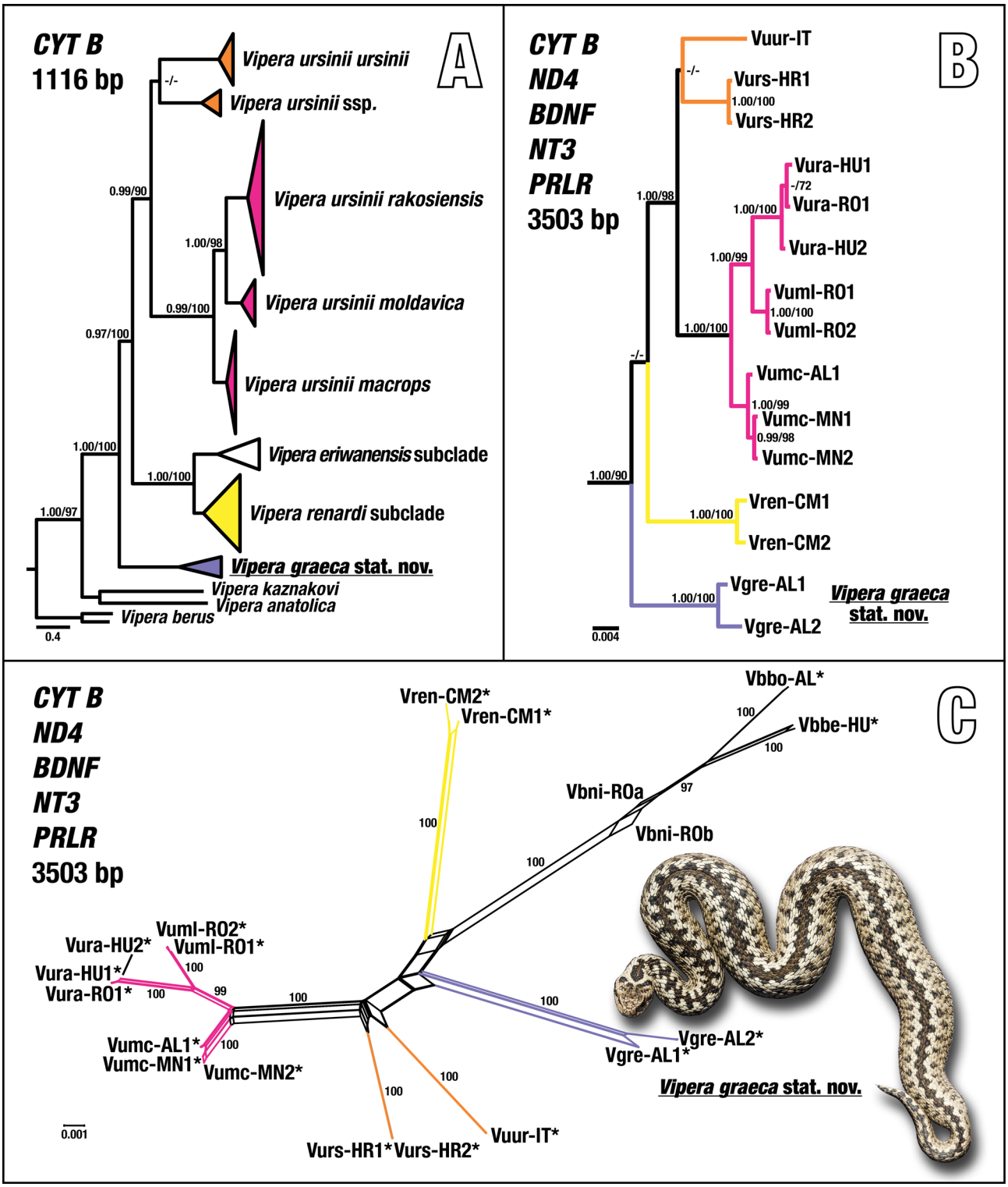

FIGURE 2. (A) Current mitochondrial Bayesian phylogenetic hypothesis of *Vipera ursinii–renardi* complex based on *CYT B* dataset of Ferchaud *et al*. (2012) and Zinenko *et al*. (2015); (B) Phylogenetic reconstruction of the concatenated dataset (mtDNA+nDNA genes) obtained in MrBayes/Maximum likelihood (see Table 1). Sequences of *Vipera berus* (Vbbe-HU, Vbbo-AL, Vbni-RO) included as outgroup are not shown. Bayesian posterior probabilities/bootstrap pseudoreplicates are shown at nodes; (C) SplitsTree phylogenetic network (Huson & Bryant 2006) of the dataset for five mitochondrial and nuclear loci sequenced in the present study using the neighbornet algorithm. Asterisks in Fig. 2C indicate both phased sequences in one branch. Numbers along the edges are the bootstrap support values from 1000 replicates. The scale bar indicates one substitution per one hundred nucleotide positions. Taxon names of the phylogenetic network correspond with the Table 1. Inset shows a male Greek Meadow Viper from Dhëmbel Mountains, Albania.

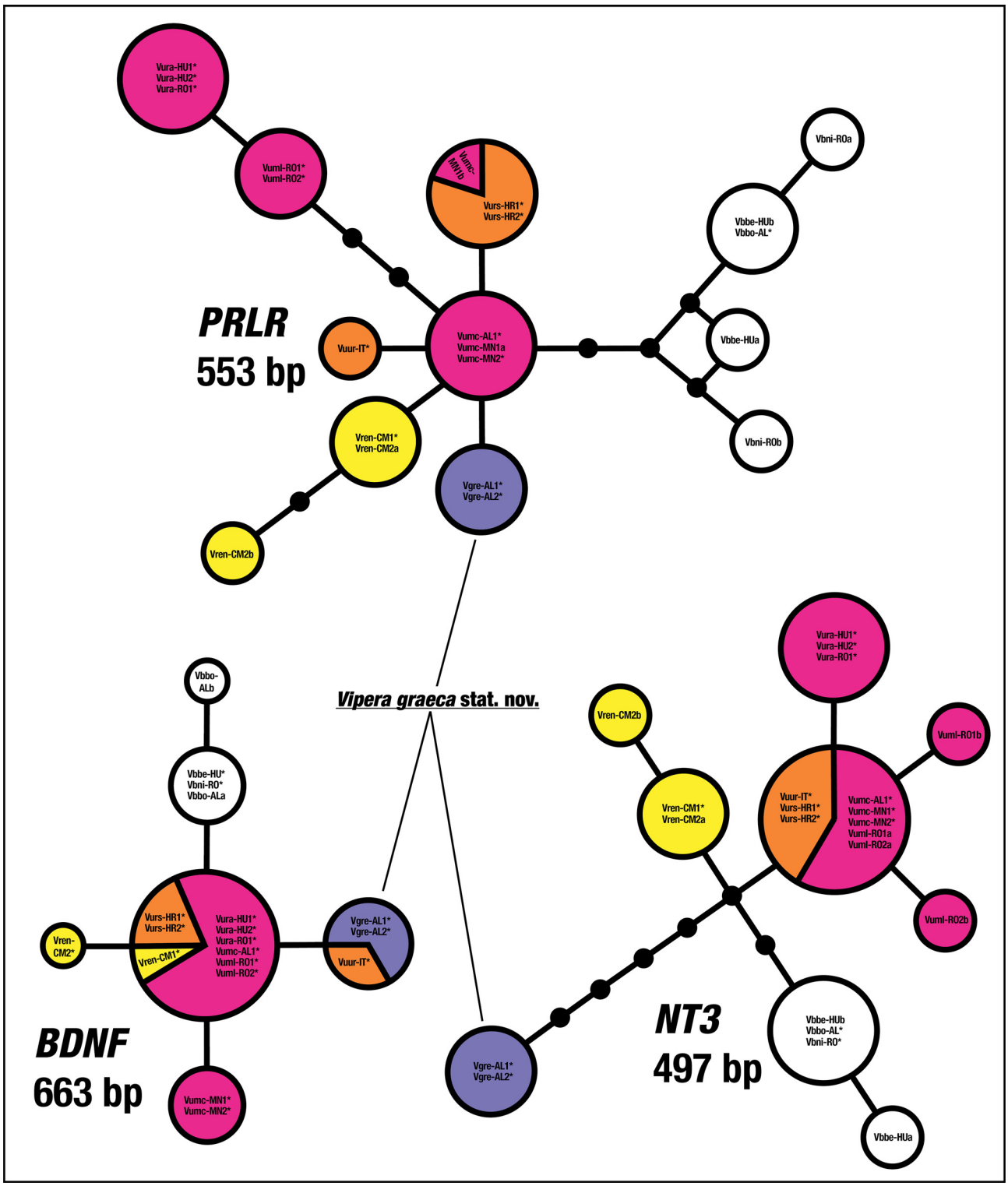

FIGURE 3. Nuclear allele networks of the three analysed nuclear loci. Circle sizes are proportional to the number of samples/sequences, small black circles indicate hypothetical haplotypes (alleles). This figure is published in colour in the online version, the colour of the circles in the network corresponds to the colour of mitochondrial lineages in Fig. 2A.

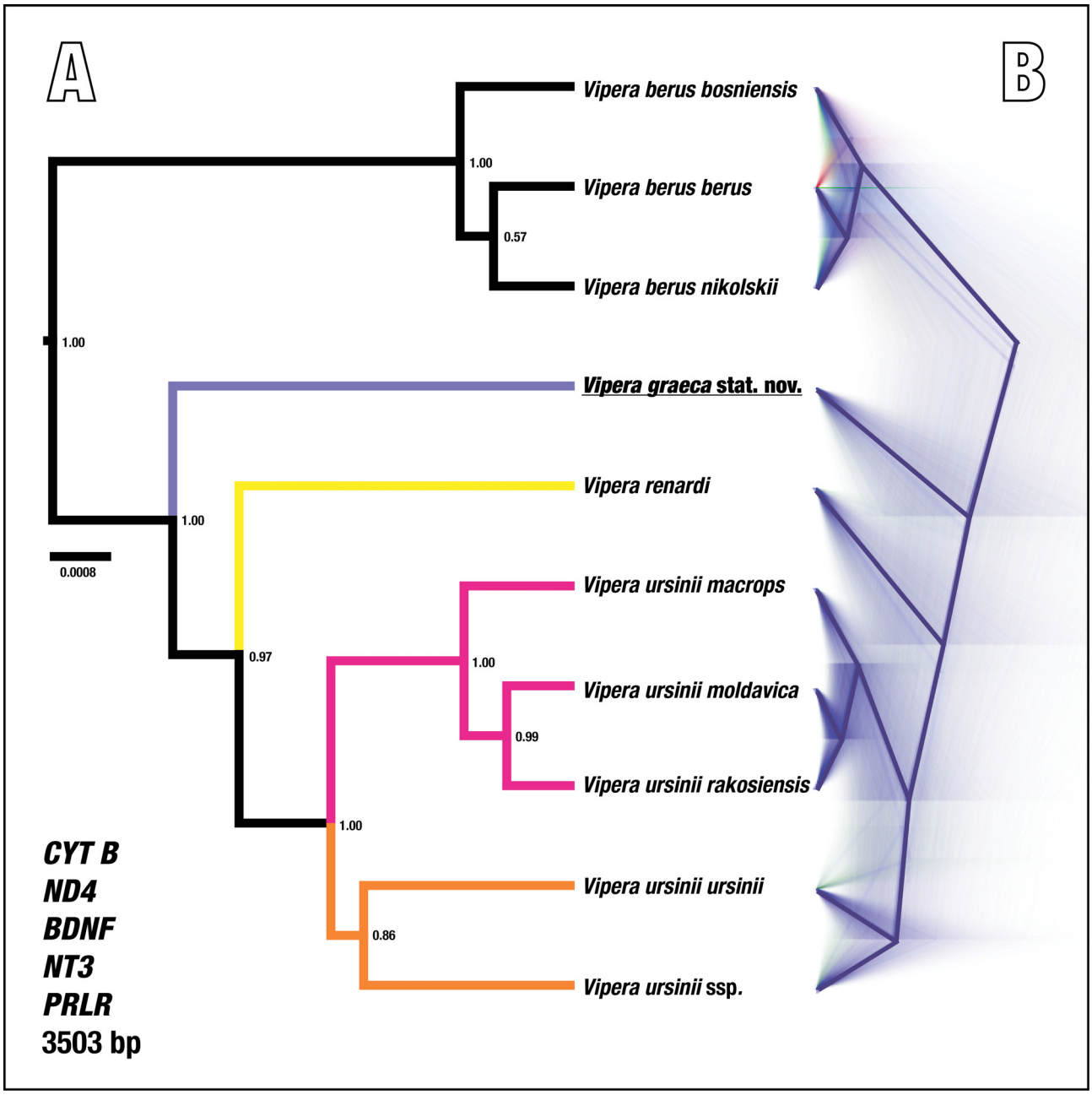

FIGURE 4. Species tree of the *Vipera ursinii–renardi* complex (with *V. berus* as outgroup) as inferred in *BEAST based on two mitochondrial and three nuclear loci (A); species-tree cloudogram of the complex based on 27 000 post-burn-in trees resulting from 3 runs of *BEAST, each producing 10,000 trees from which 10% was discarded as burn-in. Higher colour densities represent higher levels of certainty. Maximum clade credibility tree is superimposed upon the cloudogram in bold violet (B). Values of posterior probabilities are given. This figure is published in colour in the online version, the colours of the branches correspond with the colour of mitochondrial lineages in Fig. 2A.

Systematics

In accordance with the evolutionary (Wiley 1978), general lineage (de Queiroz 1998), integrative taxonomic (Miralles *et al.* 2010), phylogenetic (Cracraft 1983) and genetic species concepts (i.e. genetic species is a group of genetically compatible interbreeding natural populations that is genetically isolated from other such groups; Baker & Bradley 2006), we propose a full species rank for the Greek Meadow Viper to resolve the polyphyly in the *Vipera ursinii–renardi* complex. This is supported by morphology, distribution, ecology and genetics (i.e. multilocus approaches) as is described below.

***Vipera graeca* Nilson & Andrén, 1988 stat. nov.**
Greek meadow viper

Vipera ursinii graeca Nilson & Andrén, 1988
Vipera macrops graeca Welch, 1994: 123

Holotype. Göteborg Natural History Museum, GNM Re. ex. 4942. Leg. Nilson & Andrén 1988.

Paratypes. GNM Re. ex. 6823 (six newborn), GNM Re. ex. 6849 (ZIG 146), GNM Re. ex. 6850 (ZIG 147), GNM Re. ex. 6851 (ZIG 142) + GNM ZIG 145. Leg. Nilson & Andrén 1988.

Terra typica. Peristeri, Lakmos Mountains in the central Pindos mountain range, 1900 m altitude, Greece (Nilson & Andrén 1988).

Morphological Diagnosis. This taxon differs from all other members of *V. ursinii–renardi* complex by having the following combination of morphological characters (Nilson & Andrén 1988; Nilson & Andrén 2001; Mizsei *et al.* 2016): small body size (for males a snout to vent length (SVL) max. 40.6 cm, and tail length is 5.4 cm, and for females a SVL max. 44.3 cm, and tail length is 4.1 cm); non-bilineate body ground colour pattern; white or bright brownish-grey ventral colour; no dark spots on labial, lateral and dorsal sides of head except occipital and postorbital stripes; dorsal zigzag pattern tagged with pointed corners at windings, or consisting of a narrow vertebral line only; 45–58 dorsal windings; nasal divided into two plates or united with nasorostralia; rostral as high as broad; 2–8 loreals; 13–20 circumoculars; upper preocular not separated from nasal; 7–20 crown scales; more fragmented parietals; 12–15 supralabials (sum of right and left sides); first three supralabials two times larger than the following ones; third supralabial below orbit; 14–19 sublabials (sum of right and left sides); 3–5 mental scales; early dorsal scale row reduction; 120–129 ventrals for males, 119–133 ventrals for females; lowest number of subcaudals in the complex: 21–29 subcaudals for males, 13–26 subcaudals for females.

Molecular Diagnosis. The works of Ferchaud *et al.* (2012) and Zinenko *et al.* (2015) showed divergence of *Vipera graeca* **stat. nov.** based on mtDNA datasets. Its distinction is supported by phylogenetic position (basal taxon for all other species of the *ursinii-renardi* complex), time of divergence (the Middle Pliocene) and value of uncorrected pairwise p–distances (4.5% in view of *ursinii* clade) as is defined by Ferchaud *et al.* (2012). All our analyses based both on mitochondrial and nuclear loci support these results. Specimens in the study of Ferchaud *et al.* (2012) originated from Stavros area in the Vardoussia Mts., Greece), but in the present study we used samples from Albania. The specimens used here share the same *CYT B* and *ND4* haplotypes as the previously analysed Greek samples of Ferchaud *et al.* (2012). A single *ND4* haplotype is presented by the southernmost (Stavros area, Vardoussia Mts., Greece) and the northernmost populations (Tomorr Mts., Albania; Mizsei *et al*. 2016). Therefore, a single haplotype is very likely to be present throughout most of the distribution area of this species. Because the sequence variability of the nDNA regions is, in general, much less variable than mtDNA (Townsend *et al.* 2008), we could use specimens originated from another locality than the type locality as they represent the same phylogenetic pattern and position.

Distribution. The species occurs in the subalpine meadows of the Hellenides mountain system of southern Albania and central Greece (Dimitropoulos 1985; Nilson & Andrén 1988; Nilson & Andrén 2001; Korsós *et al*. 2008; Mizsei *et al.* 2016). These localities are Koziakas, Lakmos (Peristeri; type locality), Metsovon, Oiti, Tsouka Karali, Tzoumerka (Athamanika), Tymfristos, Vardoussia (including Stavros area) mountains in Greece, and Dhëmbel, Llofiz, Lunxhërisë, Griba, Nemerçkë (crossborder mountain, called Nemertzika in Greek), Shëndelli, Tomorr and Trebeshinë mountains in Albania. The entire distribution is extremely fragmented and each mountain population is completely isolated by a large matrix of unsuitable habitat for the taxon consisting of deep valleys and plains.

Ecology and habitats. A mosaic of open or closed grass and shrub communities formed on limestone characterizes the main habitats of the taxon. Annual mean temperatures are about ~6°C, and the meadows are partially covered by snow until early summer (May-June. South-facing slopes are usually more open and rocky than north-facing slopes. Different species of *Festuca*, *Poa* and *Sesleria* dominate the open grasslands, and characteristic shrubs are *Juniperus sabina*, *Daphne oleoides* and *Astragalus creticus*. Most of the observed vipers were found close to shrubs or stone piles in south-facing habitat patches. The diet of the species consists mainly of Orthoptera (97%) species, of which *Stenobothrus rubicundulus*, *Platycleis* sp., *Decticus verrucivorus* is the most frequent prey (Mizsei *et al*. in prep.). The abundance of Orthopterans is high from June to September (Lemonnier-Darcemont *et al*. 2015.). Known predators of the species are *Vulpes vulpes*, *Falco tinnunculus* and *Circaetus gallicus*.

Discussion

Our results of analysing two mitochondrial and three nuclear gene fragments support the distinct position of *Vipera graeca* **stat. nov.** first observed by the mtDNA results of Ferchaud *et al.* (2012). This was found in all four analytical approaches used, i.e. gene phylogenetic reconstruction, phylogenetic network, allele (haplotype) networks and coalescent species tree. Furthermore, the results support the uniqueness of the taxonomically unrecognized meadow viper lineage from Croatia, which needs a formal description and further investigation. The amendation of the subspecies status for *V. graeca* **stat. nov.** may influence the taxonomy of other taxa within *V. ursinii–renardi* complex, and call to attention the revision of the taxonomic entities in this geographically and evolutionary polytypic complex. It is important to use multiple loci in molecular approaches, and/or integrate other morphological approaches to taxonomical assessments (e.g. using hemipenes or skull morphology) to resolve complex relationships among taxa of meadow vipers.

The meristic morphology of *V. graeca* **stat. nov.** is very similar to *V. u. macrops*, as well as to other montane populations of *V. ursinii* s. l. Therefore it is not surprising that it was first described as a subspecies of *V. ursinii* when it was discovered (Nilson & Andrén 1988). Albeit morphological characteristics function for the determination of the taxon *V. graeca* **stat. nov.**, there is a conflict between morphological and molecular evidence (compare Nilson & Andrén 2001 and Ferchaud *et al.* 2012). Among European reptiles, incongruence between morphological and molecular data is common in their phylogeny (see Pisani *et al*. 2007; Assis 2009; Gvoždík *et al*. 2010; Kindler *et al*. 2013 and references therein). Based on the hypothesis if suggested colonization and diversification of the *V. ursinii–renardi* complex (Ferchaud *et al.* 2012; Zinenko *et al*. 2015), the shifts of steppe like grassland habitats following climatic oscillations were not only latitudinal dispersion, but in the Hellenides mountain complex also vertical (altitudinal) shifts. Thus, the meadow viper ancestors in the Balkans dispersed less compared to other lineages, and the morphological similarity could be explained by the presence and higher prevalence of plesiomorphic characteristics in taxa close to the radiation centre.

The extant distribution of *V. graeca* **stat. nov.** is severely fragmented (Mizsei *et al*. 2016), because the populations are currently in interglacial refugial "sky-islands" of the Pindos mountains, surrounded by a sea of coniferous/deciduous forests that make up the unsuitable habitat in the deep valleys below. This viper is threatened because populations are completely isolated from each other in small patches, intentionally killed by local people and are at risk over extinction vortices. Populations are probably very small due to microhabitat preferences, highly susceptible to inbreeding depression and genetic drift. Climate change is a contemporary concern as warming temperatures in the Mediterranean threaten the species' habitat by tree encroachment and/or secondary submediterranean grasslands (Kunstler *et al.* 2007). Following the IUCN Red List criteria the conservation status of this taxon should be Endangered (B2abiii). Most suitable habitats are used as sheep, goat or cattle pastures, and overgrazing has a direct negative effect on habitat structure in these meadows (Papanastasis *et al.* 2002). Our study not only helped resolve some uncertainties within the *V. ursinii–renardi* complex but significantly contributes to assessing the conservation status of *V. graeca* **stat. nov.**, laying a platform for which future conservation efforts may be initiated for this region.

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