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# Mitochondrial phylogeny shows multiple independent ecological transitions and northern dispersion despite of Pleistocene glaciations in meadow and steppe vipers (*Vipera ursinii* and *Vipera renardi*)



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## ABSTRACT

The phylogeny and historical demography of small Eurasian vipers of the *Vipera ursinii* and *V. renardi* complexes were studied using mitochondrial DNA sequences analysed with Bayesian inference, Maximum Likelihood and Maximum Parsimony approaches, and mismatch distributions. Diversification in the group resulted from an initial dispersion in the later Pliocene – Pleistocene in two directions: north-westwards via the Balkans (*V. ursinii* complex) and north-eastwards from Asia Minor via the Caucasus (*V. renardi* complex). An independent, comparatively recent transition occurred from montane habitats to lowland grasslands in different mitochondrial lineages during the Late Pleistocene, when representatives of the both complexes had reached lowland steppes to the north. Effective population size showed clear signs of rapid growth in eastern *V. renardi*, triggered by colonization of vast lowland steppes, but in western *V. ursinii* complex grew during the Last Glaciation and experienced stabilization in Holocene. Expansion and population growth in lowland lineages of *V. renardi* was not strongly affected by Pleistocene climatic oscillations, when cold, dry conditions could have favoured species living in open grasslands. The high diversity of closely related haplotypes in the Caucasus and Tien-Shan could have resulted from repetitive expansion-constriction-isolation events in montane regions during Pleistocene climate fluctuations. The mitochondrial phylogeny pattern conflicts with the current taxonomy.

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## 1. Introduction

Pleistocene climatic fluctuations profoundly affected the distributions and structure of most European species (Hewitt, 1999; Taberlet et al., 1998; Nieto, 2011). Traditional biogeographic

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scenarios suggest that extinction of thermo-sensitive taxa occurred during cold stages of climatic cycles in northern Europe and persisted in southern refugia. Northern areas were covered by an ice sheet and many species could not inhabit the severe periglacial zone. Recolonization of northern areas occurred during warm stages (Ursenbacher et al., 2006b; Joger et al., 2007; Barbanera et al., 2009). Accumulation of data from different taxa have resulted in more complex picture how climatic oscillation and glaciations cycles shaped distribution and genetic diversity across European species (Stewart et al., 2010; Nieto, 2011). For example, cold-tolerant species could persist in refugia located outside of traditionally recognized southern refugia (Taberlet et al., 1998; Hewitt, 1999; Ursenbacher et al., 2006a; Joger et al., 2007; Randi, 2007; Zeisset and Beebe, 2008). Pre-Pleistocene radiations also occurred in many thermophilic European species (Guicking et al., 2006; Joger et al., 2007; Guicking et al., 2009; Recuero et al., 2012).

Changes of entire Eurasian biomes were caused by both shifts of mean temperatures and a complex suite of environmental factors, including precipitation and continentality (duration of seasons, differences in temperature and humidity between summer and winter etc.), among other factors (Velichko and Spasskaya, 2002). Xerophytic grasslands (steppes) and their species are frequently distributed in areas of low mean annual temperatures, but warmer in summer than temperate forests. Their distribution is determined by an equal or slightly negative balance of precipitation and evaporation. Intervals of major glacial cooling were drier globally (Suc et al., 1999; Velichko, 1989; Velichko and Spasskaya, 2002). Therefore, the largest distribution of steppes and steppe-like open landscapes occurred at times of dry glaciations (Velichko, 1989; Artyushenko and Turlo, 1989). Warm interglacial periods, such as now, favoured the expansion of forests and together with sea transgressions in the past could have led to the shrinkage and fragmentation of steppes, as exemplified by regions near the northern Black Sea and Caspian Sea (Blagovolin et al., 1982; Artyushenko and Turlo, 1989; Velichko and Spasskaya, 2002).

The mitochondrial phylogeny projected on a geographic dimension (genogeography *sensu* Serebrovsky, 1928; phylogeography *sensu* Avise et al., 1987) can yield insights into how Pleistocene climatic cycling impacted the distribution and biogeography of the Eurasian steppe biota. Because mitochondrial DNA (mtDNA) is clonally inherited and passed exclusively through matrilineal lines in most animals, historical patterns of dispersal are not obfuscated by genetic recombination and gene sorting, which occurs in the nuclear DNA (nDNA) genome. Consequently, mitochondrial phylogenies can track the response of species to climate changes and yield predictions into future reactions at least from the perspective of female dispersal. Analyses of mtDNA sequence tell only one part of a potentially more complex story (William et al., 2004; Godinho et al., 2008), yet they provide valuable insights into the evolutionary history of species, including consequences of habitat changes, adaptation, impact of climate fluctuations and dispersal. Biome-restricted indicator species serve as ideal models for such investigations.

Vipers of the *Vipera renardi* complex mostly occupy lowland steppes from central Ukraine eastwards across southern Russia and Kazakhstan to western China. *Vipera renardi* is the indicator species of snakes of the continuous Eurasian steppe belt. The range of *V. r. renardi* covers great homogenous territories of steppe almost without disruption (Bannikov et al., 1977; Nilson and Andr n, 2001) and coincides with steppe zone itself. In contrast, European *V. ursinii* mostly occurs in montane grasslands. Historical scenarios on the origin and colonization in steppe and meadow vipers fall into two categories: (a) periodic expansion and subsequent fragmentation of the northern lowland range during the Pleistocene along with isolation and speciation in southern montane refugia (Nilson and Andr n, 2001; Tuniyev et al., 2010); or

(b) having an earlier montane southern origin with a later dispersion to the north (Nilson and Andr n, 2001; Kukushkin, 2009).

Meadow and steppe vipers of the *V. ursinii-renardi* group are a morphologically and ecologically well defined assemblage. Belonging to genus *Vipera* Laurenti, 1768, sometimes the group is assigned to subgenus *Acridophaga* Reuss, 1927 (Nilson and Andr n, 2001) or placed in subgenus *Pelias* Merrem, 1820. Morphology-based reviews since the mid-20th century have been inconclusive with respect to the recognition of taxa, especially in the poorly explored Asian and Caucasian regions (Kramer, 1961; Saint Girons, 1978; Dely and Stohl, 1989). Although these vipers were also among the first reptiles to be studied taxonomically using molecular data (Joger et al., 1992; Herrmann et al., 1992), a molecular phylogeny remains elusive. Until recently, few representatives were included in phylogenetic (Kalyabina-Hauf et al., 2004; Garrigues et al., 2005) and population genetic studies (Ujvari et al., 2005; Ferchaud et al., 2010). Ferchaud et al. (2012) and Gvozdkik et al. (2012) focused on the *V. ursinii* complex. They evaluated few samples of the *V. renardi* complex and in doing so they neither covered taxonomic diversity nor geographic range of *renardi* complex, especially in the Caucasus.

The group subdivides into two widely accepted complexes. The *Vipera ursinii* complex of meadow vipers consists of *Vipera ursinii ursinii* (Bonaparte, 1835) and the Balkan taxa *Vipera ursinii macrops* Mehely, 1911, *Vipera ursinii rakosiensis* Mehely, 1893, *Vipera ursinii graeca* Nilson and Andr n, 1988, and *Vipera ursinii moldavica* Nilson, Andr n et Joger, 1993. The eastern steppe vipers, i.e. the *Vipera renardi* complex, consists of *Vipera renardi* (Christoph 1861), with the subspecies *V. r. bashkirovi* Garanin et al. (2004), *V. r. puzanovi* Kukushkin (2009), *V. r. tienshanica* Nilson et Andr n 2001, and *V. r. parursinii* Nilson et Andr n 2001, as well as *Vipera eriwanensis* (Reuss 1933), *Vipera ebneri* Kn pfler et Sochurek 1955, *Vipera lotievi* Nilson et al. (1995), *Vipera altaica* Tuniyev et al. (2010) (Nilson and Andr n, 2001; Joger and Dely, 2005; Dely and Joger, 2005; Kalyabina-Hauf et al., 2004). Taxonomy is not without controversy. Whereas Joger and Dely (2005) reduced *Vipera lotievi* to being a subspecies of *V. renardi*, some authors continue to recognize the species (Tuniyev et al., 2009, 2011). *Vipera anatolica* Eiselt et Baran 1970 from southwestern Turkey was considered a subspecies of *V. ursinii* (Billing, 1985) or a species of the *renardi* complex together with *V. renardi* and *V. eriwanensis* (Nilson and Andr n, 2001). Kalyabina-Hauf et al. (2004) resolved *V. anatolica* as the sister-group of the *ursinii*, *renardi* and *kaznakovi* complexes based on analyses of mtDNA sequences. Recently described taxa include *V. r. bashkirovi* (Garanin et al., 2004), *V. r. puzanovi* (Kukushkin, 2009), the “Altai taxon of *V. renardi*” of Nilson and Andr n (2001) as *V. altaica* (Tuniyev et al. (2010)) and morphologically similar to *V. eriwanensis* population of steppe viper from  amaxi as *Vipera shemakhensis* (Kukushkin et al., 2012; Tuniyev et al., 2013). The phylogenetic positions of these new taxa remain unknown.

The primary aim of our work is to reconstruct the mitochondrial phylogeny of *ursinii-renardi* group, outline distribution of mitochondrial clades and give insight into the dispersal history and historical demography of populations on the background of climatic oscillations of Pleistocene, landscape rearrangements and the transition between highland meadows and lowland steppes.

## 2. Material and methods

### 2.1. Sampling and molecular protocols

We used 429 vipers samples, taken from specimens from collections of the Zoological Institute of Russian Academy of Sciences, St. Petersburg, Russia (ZISP); Sochi National Park, Sochi, Russia (SNP), Natural History Museum Gothenburg, Sweden (NMG), Zoological

Museum of State Moscow University, Moscow, Russia (ZMMSU), The Museum of Nature at V. N. Karazin Kharkiv National University, Kharkiv, Ukraine (MNKNU), Zoological Museum of National Natural History Museum, Kiev, Ukraine (ZMNHMK); stored in tissue collections of Zoological Institute of Kazakh Academy of Sciences, Almaty, Kazakhstan (ZIA); State Natural History Museum, Braunschweig, Germany (SNMB); Royal Ontario Museum, Toronto, Canada (ROM); or from alive snakes in Tula Regional Exotarium (TE) and animals in the wild (Supplementary Table 1). No animals were sacrificed for this study. Tissue samples were stored in 95% ethanol in cold conditions. Exuvia were kept in individual bags, in dry, cool conditions. Genomic DNA was extracted from muscle or exuvia using DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions.

Primers and PCR conditions were adapted from Stümpel (2012). Initially, we amplified a 1159 bp fragment of the mtDNA gene encoding cytochrome *b* (*Cytb*) and sequenced fragment lengths up to 887 bp for all samples. Additionally, we amplified and sequenced the complete *Cytb* and 990 bp fragment of the gene encoding cytochrome c oxidase I (*COI*; Entrez: *MT-COI*; 1605 bp) with reverse or internal primer for 42 selected representatives of the main clades, identified by preliminary analysis. The approach after aligning and trimming ends gathered up to 2021 bp in total. PCR was carried out using the following primers (Stümpel, 2012): *Cytb\_F1* tgaggcctgaaaaccaccgttg (position of last bp on 3' end corresponds to 14971 bp of *M. xanthina* reference genome) and *Cytb\_RC* agcttcgttttacaagaacggtgc (position of last bp on 3' end corresponds to 16125 bp of *M. xanthina* reference genome), *COI\_F1* acagttaccgcttataatcagcca (position of last bp on 3' end corresponds to 6246 bp of *Montivipera xanthina* reference genome) and *COI\_R1* atagtgggcagttaaagtctgtctc (position of last bp on 3' end corresponds to 7852 bp of *M. xanthina* reference genome). Except *COI\_R1* PCR primers and two additional internal primers *Cytb\_F8* gcagcaacagtaatacacaactcc (position of last bp on 3' end corresponds to 15413 bp of *M. xanthina* reference genome) and *COI\_F3\_vip* cactcaggccatcagtagacct (position of last bp on 3' end corresponds to 6688 bp of *M. xanthina* reference mitochondrial genome) were used for sequencing. Dye terminator cycle sequencing was set up according to suppliers' instructions (DTCS Quick Start Kit, Beckman Coulter) in a two-step thermal reaction with 30 cycles of 96 °C 20 s, 60 °C 4 min. Products were purified via spin columns (QIAquick PCR Purification Kit) and run on Beckman Coulter CEQ 8000 sequencing apparatus or sequenced by a commercial sequencing service.

For amplifying target genes, the TaKaRa Ex Taq™ PCR reaction system was used, containing 2.5 µl 10X Buffer, 2 µl dNTP Mix, 2.5 U enzyme, 1 µl of 10 pM primer each, 1 µl genomic DNA, filled with dH<sub>2</sub>O to 25 µl volume in total. PCR conditions for *Cytb* included an initial denaturation step at 95 °C 2 min, followed by 35 cycles of 94 °C 40 s, 60 °C 40 s, 72 °C 1.1 min and a final extension at 72 °C for 5 min. PCR reaction for *COI* started with an initial denaturation step at 95 °C 2 min followed by 35 cycles of 94 °C 45 s, 57 °C 45 s, 72 °C 2 min and a final extension at 72 °C for 5 min. Products were cooled down and stored until use at 8 °C.

Haplotypes identified in our data were uploaded to GenBank, for accession numbers see Supplementary Table 1. Additional sequences of Old World vipers for the analyses were obtained by us (*Bitis arietans* (Merrem, 1820), *Daboia mauritanica* (Duméril et Bibron, 1848), *Macrovipera lebetina* (Linnaeus, 1758), *Montivipera raddei* (Boettger, 1890), *Montivipera xanthina* (Gray, 1849), different representatives of *Vipera kaznakovi* Nikolsky, 1909 complex, *Vipera seoanei* Lataste, 1879, *Vipera berus* (Linnaeus, 1758), *Vipera anatolica* (Eiselt et Baran, 1970)) or retrieved from GenBank (*Causanus defilippii* (Jan, 1862) – Castoe et al., 2009; *Daboia russelii* Shaw et Nodder, 1797 – Chen N., Fu X. Y, unpublished). Haplotypes of Cytochrome B of the *ursinii-renardi* group, absent in our data set,

were retrieved from GenBank as well (Ferchaud et al., 2012; Gvozdk et al., 2012) (Supplementary Table 1).

## 2.2. Sequence alignment and phylogenetic analyses

Forward and reverse or forward and internal sequences of the same gene were edited and assembled using SEQUENCHER (Gene Codes). Genes sequences obtained by us and retrieved from GenBank were aligned using ClustalW (Thompson et al., 1994) implemented in Bioedit 7.0.9 (Hall, 1999) and checked by eye. Extra sequences representing the same haplotype were removed from analyses resulting to the final alignment of 122 sequences of different length. Adding of taxa with missing data and partial overlap is considered to cause minor problems to phylogeny reconstruction and in the same time could add valuable information to it (Queiroz and Gatesy, 2006). Same alignment was used in subsequent analyses unless other is specified in the text. General characterization of DNA variation and McDonald-Kreitman test for neutrality were done in DnaSP 5.10 (Librado and Rozas, 2009), partition of genetic variation was analysed by AMOVA in Arlequin 3.5.1.3.

Numerous studies have revealed a widespread phenomenon of integration of mtDNA to a nucleus in different organisms (Bensasson et al., 2001; Richly and Leister, 2004; Leister, 2005) including reptiles (Podnar et al., 2007). A pooled analysis of authentic mtDNA sequences and nuclear mitochondrial pseudogenes (NUMTs) can mislead phylogenetic reconstructions due to their different evolutionary history and posed serious challenge to mitochondrial based studies (Song et al., 2008). Several measures were proposed to control NUMTs (Song et al., 2008). Since our study objects are rare and in most of the cases we could not get access to tissues reach in mitochondria, this type of control was not suitable for us. However, the presence of NUMTs in our data set is unlikely since (1) amplicons of sample DNA were visualized on a gel and absence of double bands indicated on one product of PCR reaction, (2) all chromatograms were checked by eye and did not contain double peaks, (3) sequences were in reading frame and did not have indels or stop codons (Song et al., 2008; Collura and Stewart, 1995), (4) topology of trees built in preliminary analysis (not shown, available upon request) with both genes independently were identical. Additionally, length of amplified fragments is longer than average size of NUMTs (Leister, 2005).

Phylogenetic trees were reconstructed using Maximum Parsimony (MP), Bayesian inference (BI) and Maximum likelihood (ML). To identify the most appropriate partition of data and model of sequence evolution for the dataset, we used PartitionFinder 1.0.1 (Lanfear et al., 2012), and selected the GTR+G+I and unpartitioned dataset favoured under the Bayesian Information Criterion (BIC = 28473.73; lnL = -13281.64; I = 0.53,  $\alpha$  = 1.01). Estimates of evolutionary divergence over sequence pairs between groups, average evolutionary divergence over sequence pairs within groups and estimates of net evolutionary divergence between groups of sequences were calculated in Mega 6 (Tamura et al., 2013).

For BI, we used MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) and treated both genes as one partition under the GTR + I + G model. We ran two independent analyses with one cold and three heated chains (MC<sup>3</sup>) for 10 million generations sampling every 500th generation. The analysis was repeated under the same settings. Trees of both runs were subsequently combined to a single majority-rule consensus tree after discarding the first 25% of the trees as burnin. Convergence was estimated in Tracer 1.5.0 (Rambaut and Drummond, 2007) and observed with the convergence diagnostic parameters (standard deviation of the partition frequencies (Stdev(s)), and the potential scale reduction factor

(PSRF)) implemented in MrBayes. Maximum Parsimony tree was calculated using PAUP\*4.0b10 (Swofford, 2001) with heuristic search of best tree and tree-bisection-reconnection Branch-swapping algorithm. For ML, we used PhyML 3.0 (Guindon and Gascuel, 2003), under the GTR model with six substitution rate categories and 1000 non-parametric bootstrap replicates.

African Viperinae are well known to be the sister group of Eurasian Viperinae (Lenk et al., 2001; Wüster et al., 2008). In both of our phylogenetic analyses, African *Bitis arietans* (Merrem, 1820) and *Causus defilippii* (Jan, 1862) were specified as the outgroup to Eurasian vipers. Representatives of *Daboia*, *Macrovipera*, *Montivipera* and all species of subgenus *Pelias* were also included in the phylogenetic analyses.

### 2.3. Estimation of divergence times

The molecular clock hypothesis (Zuckerklund and Pauling, 1962, 1965) allowed the dating of branching events. Biologically realistic models of molecular dating were used to infer divergence times (Wertheim et al., 2010). Calibrations at internal nodes usually have been based on the largely incomplete and biased fossil record (Lieberman, 2002; Hedges and Kumar, 2004), and not without problems. Fossil calibration errors have mainly owed to five factors (Gandolfo et al., 2008): (1) fossil preservation, (2) taxonomic assignment of the fossil, (3) identification of fossil homologues, (4) sampling effort, and (5) fossil age determination. Identifications at the species-level have proven difficult (Padian et al., 1994). Thus, calibration dates have been traditionally restricted to few model organisms (Benton and Donoghue, 2007). Most fossil vipers have consisted of isolated vertebrae whose taxonomic identification was unclear (Szyndlar and Rage, 1999). Head (2005) pointed out that ontogenetic variation in snake vertebrae is not well understood and yet the size of vertebrae has been used for taxonomic assignment of fossil vipers (Szyndlar and Rage, 1999). Hitherto the fossil record of Eurasian vipers does not provide enough verified evidence to date their cladogenesis. Thus, we used secondary calibrations of robust divergence time calculations (Table 1).

In the absence of known biases, we used reliable nodes as a secondary calibration point (Hedges and Kumar, 2004). We employed divergence time calibration points inside Viperinae, calculated using protein coding amino acid sequences of complete mt-genomes and calibration points of splits of main lineages of amniotes (Stümpel, 2012). Node ages used as secondary calibration in this paper were biogeographically validated when divergence times coincided with vicariant paleogeographic reorganisations in the circum-Mediterranean region, which shaped the radiation of Eurasian vipers (Stümpel, 2012). Thus, *Montivipera raddei* separated from *M. xanthina* during Middle Serravallian by a marine connection along the Bitilis Eastern Anatolian Fault Zones between Tethys and Paratethys (Rögl, 1999). The time-window between 9.3 and 15.9 Mya during the lower Tortonian, Serravallian and Langhian was covered by the 95% confidence interval. *Montivipera* and *Macrovipera* split because of the long-standing isolation of “Asia Minor” during Langhian between 16 and 15 Mya based on the

lithological-paleogeographic maps of Popov et al. (2004). During Burdigalian the ‘Gomphotherium landbridge’ allowed faunal exchange between African and Eurasian biota (Van der Made, 1997; Rögl, 1999; Agusti et al., 2001). The opening of the intercontinental corridor also played an important role for the dispersal of thermophilic ectothermic vertebrates (Böhme, 2003). The bicontinental distribution of *Daboia* and the divergence time for the most recent common ancestor (MRCA) of *Daboia russelii* and *D. mauritanica* strongly coincided with the emergence of the landbridge. The divergence time of the most basal calibration point corresponded with previous analyses for the MRCA of Viperinae (39.7 Mya) calculated by Wüster et al. (2008).

For molecular dating, we used the Bayesian Markov chain-Monte Carlo (MCMC) sampler in BEAST 1.7.5 (Drummond and Rambaut, 2007). The analysis was run for 200 million generations, sampling every 1000th generation; the first 25% of samples were discarded as burnin. Parameter statistics in Tracer had effective sample sizes above 250. We used Tracer 1.5.0 (Rambaut and Drummond, 2007) to analyse the posterior distribution and effective sample size of the MCMC run. To account for lineage-specific rate heterogeneity, we used a Log-normal relaxed clock model (Drummond et al., 2006) and GTR + G + I. Since our dataset includes both intra and inter-specific samples neither coalescent nor purely Yule tree models are fully applicable (Forcina et al., 2012), we run two different analyses. In the first variant of analysis we exclude most of intra-specific samples and restrict our dataset to representatives of each lineage within *ursinii-renardi* group (23 sequences, one sequence for each monophyletic allopatric group of *V. u. ursinii*, *V. u. macrops*, *V. ebneri*, *V. eriwanensis*, *V. r. renardi*, *V. lotievi* from Dagestan, *V. lotievi* from Chechnya, *V. r. tienshanica* from the western part of the distribution, *V. r. tienshanica* from the eastern part of the distribution) and specified a birth-death process for modelling the dynamical process of speciation and extinction. Oriental vipers (*Daboia*, *Montivipera*, *Macrovipera*) were constrained topologically to be monophyletic because of prior information (Stümpel, 2012). The second analysis included all samples available and coalescent tree model was specified. Substitution saturation was assessed in both genes and each codon position was evaluated independently using the software DAMBE (Xia and Xie, 2001).

### 2.4. Historical demography

To analyse past population demographics, we examined mismatch distributions (MD) (generated in DnaSP 5.10; Librado and Rozas, 2009), for the signature of demographic expansion from a small stem-group population. We assumed unimodal mismatch distributions indicated sudden range expansions (Slatkin and Hudson, 1991; Rogers and Harpending, 1992), multimodal mismatch distributions pointed to structured or diminishing population, and ragged distributions designated a stable, widespread population (Rogers and Harpending, 1992; Rogers et al., 1996; Excoffier and Schneider, 1999; Pilkington et al., 2008).

The fit of the observed data were tested against a null distribution of constant population size by calculating Fu's (1997)  $F_s$ ,

**Table 1**  
Secondary calibration points used for divergence dating in the program BEAST v1.6.1 (Drummond and Rambaut, 2007; Drummond et al., 2005). All calibration points were adjusted using normal distribution. Mean values, and standard deviations (SD) are provided along with 95% posterior confidence intervals. MRCA – most recent common ancestor.

Calibration point	Mean (Mya)	SD (Mya)	Confidence interval (Mya)	
			5%	95%
(1) <i>Montivipera raddei</i> vs. <i>M. xanthina</i>	12.60	2.00	9.31	15.89
(2) <i>Montivipera</i> vs. <i>Macrovipera</i>	15.50	1.00	13.86	17.14
(3) <i>Daboia russelii</i> vs. <i>D. mauritanica</i>	18.05	1.00	16.41	19.68
(4) MRCA of Viperinae	38.30	2.00	35.01	41.59

Ramos-Onsins and Roza's (2002)  $R_2$ , Harpending's (1994) raggedness index  $r$  and Tajima's (1989)  $D$ . Large negative values of  $F_s$  and small positive values of  $R_2$  indicated population growth. Small index values of  $r$  indicated populations experienced a sudden expansion whereas stationary or bottlenecked populations were characterized by higher values of  $r$  (Harpending et al., 1993; Harpending, 1994).

Tests and statistics were performed under a model of constant population size and in consideration of no genetic recombination. We assessed significant deviations from null distributions by generating 10,000 coalescent simulations of a neutrally evolving population. The analyses were performed for five subsets as follows: (a) the whole *renardi* complex (*V. renardi*, *V. lotievi*, *V. ebneri* and *V. eriwanensis*); (b) only northern representatives of the *renardi* complex, excluding the geographically restricted southern subclade of *V. ebneri* and *V. eriwanensis*; (c) the whole *ursinii* complex but without haplotypes of *V. u. graeca* (it was excluded from mismatch and Bayesian skyline plot calculations because of low support of clustering with the rest of *V. ursinii*, isolated southern position in the range and incomplete sampling; d) the monophyletic clade of *V. u. rakosiensis*, *V. u. macrops* and *V. u. moldavica*; and (e) the monophyletic clade of *V. u. ursinii* and *V. u. ssp.* To enlarge the number of sequences used in the analysis, we trimmed the alignment to the shortest 540 bp *Cytb* fragment, covered in all sequences, including those that were retrieved from GenBank. To check if results are robust under different modifications of input data, we run separate analyses for the first two groups using the concatenated alignment of *Cytb* and *COI* while excluding some short sequences; this reduced the number of haplotypes.

A Bayesian skyline plot (BSP; Drummond et al., 2005) implemented in BEAST was used to provide a coalescent-based estimate of effective population size ( $N_e$ ) through time. This method did not require a pre-specified parametric demographic model as a prior. BSP credibility intervals considered phylogenetic and coalescent uncertainty (Drummond et al., 2005). Substitution and clock models were unlinked. We applied the appropriate evolutionary model GTR + G + I. The mean substitution rate was estimated under a relaxed molecular clock. To scale the BSP with time (Mya), we used estimates for the MRCA of each group obtained from divergence dating. Calibration points for *renardi* (s. l.; 1.96 Mya) and the *ursinii* complex excluding *V. u. graeca* (2.18 Mya) were modelled with a normal distribution and a standard deviation of 0.1. We employed the default settings for the analyses. MCMC was run for 300 million generations, sampling every 1000th tree and the first 25% were discarded as burnin. The posterior distribution and effective sample size of the MCMC run was inspected using the program Tracer 1.5.0 (Rambaut and Drummond, 2007).

### 3. Results

#### 3.1. Phylogeny

The analysis of 420 DNA sequences resolved 104 unique haplotypes for *Cytb* (66 haplotypes of *renardi* complex and 38 haplotypes of *ursinii* complex) and 36 haplotypes of *COI* (24 haplotypes of *renardi* complex and 12 haplotypes of *ursinii* complex). The concatenated alignment of *Cytb* and *COI* consisted of 2021 aligned positions among which 957 were invariable and 205 potentially parsimony informative. Selected pairwise uncorrected  $p$ -distances between and within taxa are presented in Table 2; clade composition used in  $p$ -distance calculation is shown in Supplementary Table 2. McDonald-Kreitman test performed to alignment of 540 bp of *Cytb* sequences and *renardi* and *ursinii* complexes as "species" did not show significant deviation from neutral model. The 24.05% of genetic variation was partitioned between clades

of *ursinii* and *renardi* complexes and 75.95% within them (AMOVA:  $F_{st} = 0.2854$ ,  $p = 0.00098$ ).

MP, BI, using both MrBayes and BEAST, and ML analyses produced highly congruent trees (Figs. 1 and 2, composition of haplogroups are given in Supplementary Tables 1 and 2). As in earlier reconstructions (Kalyabina-Hauf et al., 2004; Ferchaud et al., 2012; Gvozdik et al., 2012), the clade for the *ursinii-renardi* group departed traditionally recognized *V. ursinii*, *V. renardi*, *V. lotievi* (paraphyletic with *V. renardi*), *V. eriwanensis*, *V. ebneri* but not *V. anatolica*. The entire *ursinii-renardi* group contained haplotypes of the *V. kaznakovi* complex from Russian Western Caucasus (*Vipera orlovi* Tuniyev et Ostrovskikh, 2001, *V. kaznakovi*). Its' relationships with the *ursinii* and *renardi* complexes remained uncertain because of low nodal support values. Within the *ursinii-renardi* group, the MP, BI and ML topologies (Fig. 1) supported monophyly of both *ursinii* complex (without *V. u. graeca*) and *renardi* complex. The relationships within *Vipera* were better supported by posterior probabilities than by nonparametric bootstrapping (Karol et al., 2001; Erixon et al., 2003).

##### 3.1.1. The *ursinii* complex

The additional haplotypes of Russian *V. kaznakovi* changed the previous tree topology for the *ursinii-renardi* group (Ferchaud et al., 2012), which otherwise was identical to our tree: *V. u. graeca* switched position from the sister-group of the *ursinii-renardi* group to clustering with the remaining *V. ursinii* taxa, but has low nodal support in all reconstructions (Fig. 1). The monophyletic *ursinii* complex became restricted to Europe. It has five highly supported monophyletic lineages corresponding to *V. u. macrops*, *V. u. rakosiensis*, *V. u. moldavica*, *V. u. ursinii* and a taxonomically unrecognized lineage, hereafter called *V. u. ssp.* *Vipera u. ssp.* occupies the north-western part of Dinaric Alps (Croatia, Bosnia and Herzegovina) separated by the Neretva River from the remaining populations of *V. u. macrops* (Bosnia and Herzegovina, Serbia, Montenegro, Albania, and former Yugoslav Republic of Macedonia; Fig. 3). It is genetically most similar to *V. u. ursinii*, yet the sister relationship between it and *V. u. ursinii* has a very low support (Fig. 1; see also Ferchaud et al., 2012). Samples of *V. u. macrops* from Velebit Mountain, Croatia, used by Gvozdik et al. (2012), apparently belong to *V. u. ssp.* Uncorrected  $p$ -distances vary between 1% in the case of *V. u. moldavica* and *V. u. rakosiensis* (*Cytb*, Table 2), but usually they were higher than 3% between subspecies of *V. ursinii*. Genetic distances between haplotypes inside lineages of one subspecies were usually less than 0.5%. *V. u. graeca* potentially represent sixth lineage of European clade of *V. ursinii*, however could not definitely confirm this grouping due to a low support of such clade.

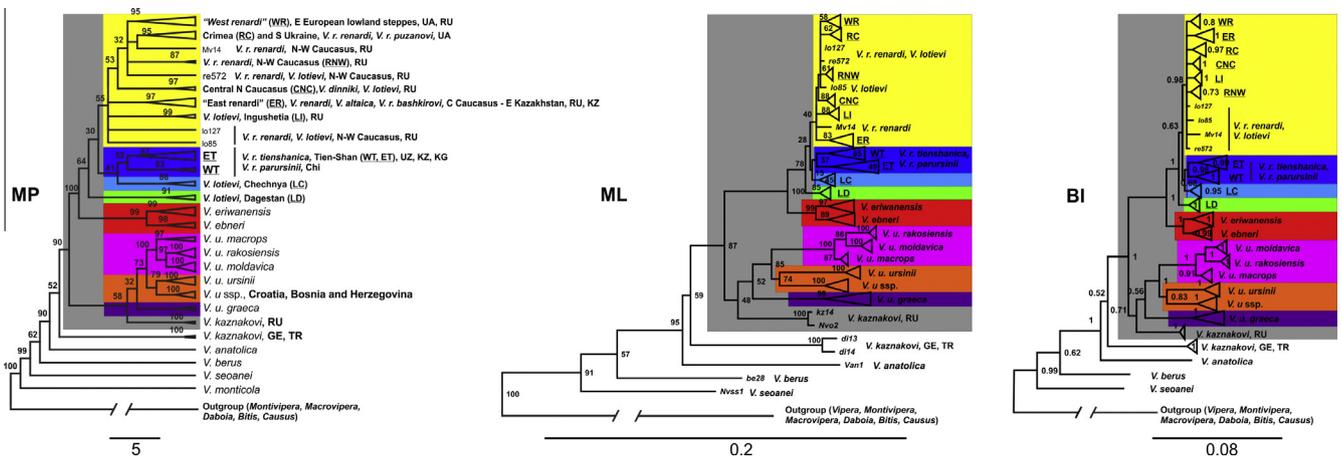
##### 3.1.2. The *renardi* complex

The *renardi* complex shows a split into two main highly supported clades: I) *V. eriwanensis-V. ebneri* clade; and II) *V. renardi* clade (including all subspecies of *V. renardi* as well as *V. lotievi* and *V. altaica*). The *V. eriwanensis-V. ebneri* lineage is further divided into *V. eriwanensis* from the Armenian upland and *V. ebneri* from Iran (Fig. 1). Other reconstructions (Ferchaud et al., 2012; Gvozdik et al., 2012) resolved the same primary mitochondrial lineages inside the *renardi* complex. *V. renardi* clade includes four monophyletic subclades with not fully resolved relationships between them (posterior probabilities lower than 0.68, bootstrap values lower than 28%): two highly restricted lineages from Eastern Caucasus comprised almost exclusively of specimens *a priori* morphologically assigned to *V. lotievi* (Fig. 1) ("lotievi Dagestan" – LD; "lotievi Chechnya" – LC from western Dagestan, including the *terra typica* of *V. lotievi* on the border of Chechnya and Ingushetia), *V. r. tienshanica*, and the rest of *V. r. renardi* (40% bootstrap and 0.98 posterior probability) covering the most of the distribution range

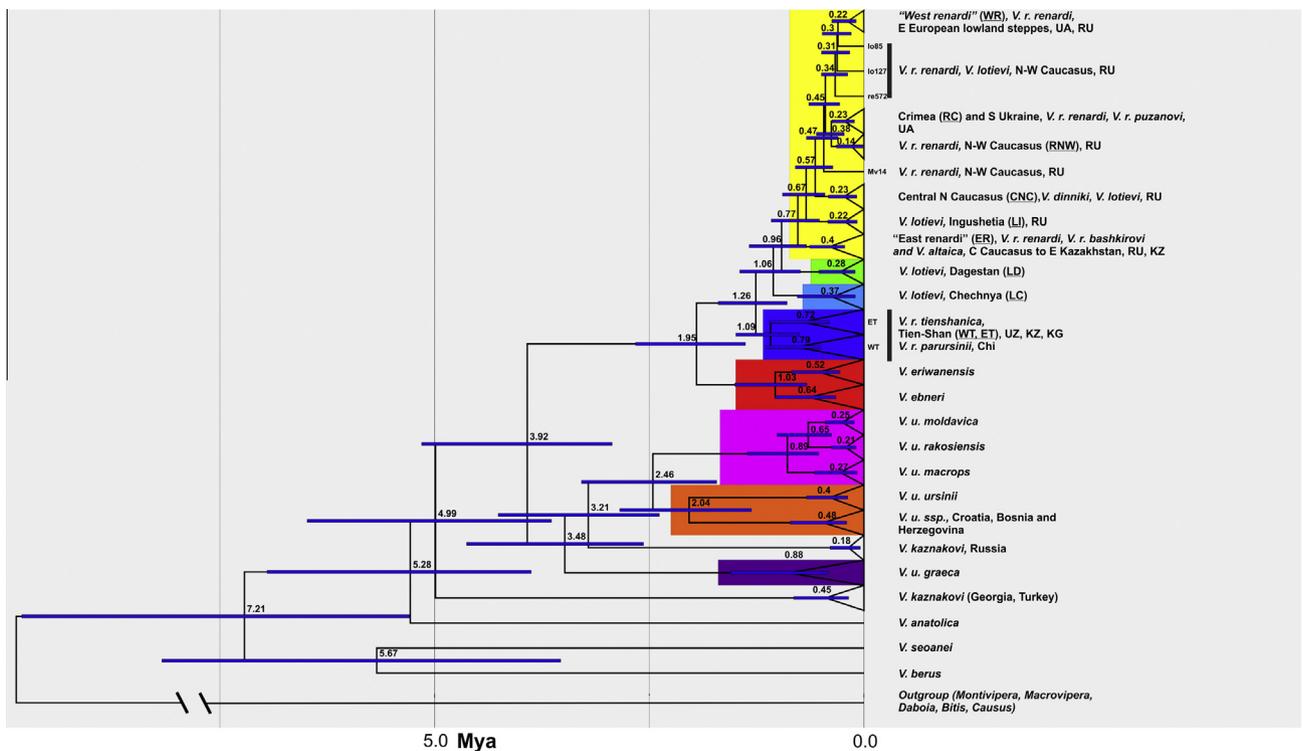
Table 2

(A) The Mean of *p*-Divergence between Groups (lower diagonal), Average Evolutionary Divergence over Sequence Pairs within Groups (in bold, diagonal) and Standart Error Estimates (upper diagonal) are shown. The analysis involved 113 nucleotide sequences of 1101 positions of Cytb. All positions with less than 20% site coverage were eliminated. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). (B) The Mean of *p*-Divergence between Groups (lower diagonal), Average Evolutionary Divergence over Sequence Pairs within Groups (in bold, diagonal) and Standart Error Estimates (upper diagonal) are shown. The analysis involved 113 nucleotide sequences of 920 positions of COI. All positions with less than 20% site coverage were eliminated. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

	Krasnodar 1 (K1), V. r. renardi (individual clades)	West V. r. renardi (WR)	V. r. renardi N-W Caucasus (NWC)	Crimea (RC)	Central N Caucasus (CNC)	V. lotievi Ingushetia (LI)	ast V. r. renardi (ER)	V. lotievi Dagestan (LD)	V. lotievi Chechnya (LC)	V. r. tienshanica W (WT)	V. r. tienshanica (ET)	V. erivanensis	V. ebneri	V. u. macrops	V. u. moldavica	V. u. rakosiensis	V. u. ursinii	V. u. ssp.	V. u. graeca	V. kaznakovi Russia	V. kaznakovi Georgia, Turkey	V. anatolica	V. berus	V. seoanei
<b>Panel A</b>																								
Krasnodar 1 (K1), V. r. renardi (individual clades)	<b>0.3</b>	0.2	0.2	0.2	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.4	0.4	0.7	0.7	0.7	0.6	0.6	0.7	0.6	0.8	0.8	0.8	0.9
West V. r. renardi (WR)	0.4	<b>0.2</b>	0.2	0.2	0.2	0.3	0.3	0.2	0.3	0.3	0.4	0.3	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.7	0.7	0.7	0.8
V. r. renardi N-W Caucasus (NWC)	0.5	0.5	<b>0.3</b>	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.4	0.4	0.7	0.7	0.7	0.7	0.6	0.6	0.6	0.6	0.8	0.8	0.8	0.9
Crimea (RC)	0.5	0.5	0.7	<b>0.2</b>	0.2	0.3	0.3	0.3	0.3	0.3	0.4	0.4	0.6	0.6	0.7	0.7	0.6	0.6	0.6	0.6	0.7	0.7	0.7	0.8
Central N Caucasus (CNC)	0.7	0.7	0.8	0.8	<b>0.3</b>	0.3	0.3	0.3	0.3	0.3	0.5	0.4	0.6	0.7	0.7	0.7	0.6	0.5	0.6	0.6	0.7	0.7	0.7	0.8
V. lotievi Ingushetia (LI)	0.8	0.8	0.9	0.9	1.0	<b>0.4</b>	0.3	0.3	0.3	0.3	0.4	0.4	0.7	0.7	0.7	0.7	0.6	0.5	0.6	0.6	0.7	0.8	0.7	0.8
East V. r. renardi (ER)	0.9	1.0	1.0	1.1	1.3	1.3	<b>0.4</b>	0.3	0.3	0.3	0.4	0.4	0.6	0.7	0.7	0.7	0.6	0.6	0.6	0.6	0.8	0.8	0.7	0.8
V. lotievi Dagestan (LD)	0.8	0.8	1.0	1.0	1.1	1.1	1.3	<b>0.2</b>	0.2	0.3	0.3	0.4	0.4	0.6	0.6	0.7	0.5	0.5	0.6	0.5	0.7	0.7	0.7	0.8
V. lotievi Chechnya (LC)	0.7	0.8	0.8	0.9	1.0	0.8	1.2	0.8	<b>0.3</b>	0.2	0.2	0.4	0.4	0.6	0.6	0.6	0.5	0.5	0.6	0.5	0.7	0.7	0.7	0.8
V. r. tienshanica W (WT)	1.2	1.3	1.3	1.3	1.5	1.5	1.4	1.3	1.1	<b>1.2</b>	0.3	0.4	0.4	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.7	0.7	0.6	0.8
V. r. tienshanica (ET)	0.9	1.0	1.1	1.2	1.3	1.3	1.2	1.1	1.0	1.3	<b>0.4</b>	0.4	0.3	0.6	0.6	0.6	0.6	0.5	0.6	0.6	0.7	0.7	0.7	0.8
V. erivanensis	1.8	1.7	2.0	2.1	2.2	2.1	2.1	1.9	1.8	2.1	1.9	<b>0.7</b>	0.3	0.7	0.7	0.7	0.6	0.6	0.6	0.6	0.8	0.8	0.7	0.8
V. ebneri	1.6	1.5	1.8	1.8	1.9	2.0	1.8	1.8	1.7	2.0	1.7	1.4	<b>0.5</b>	0.6	0.7	0.7	0.6	0.6	0.6	0.6	0.7	0.7	0.7	0.8
V. u. macrops	4.5	4.2	4.4	4.4	4.4	4.7	4.6	4.3	4.2	4.5	4.6	4.9	4.6	<b>0.2</b>	0.3	0.3	0.6	0.5	0.6	0.6	0.7	0.7	0.7	0.8
V. u. moldavica	4.8	4.6	4.7	4.8	4.7	5.1	4.9	4.7	4.6	4.8	4.9	5.6	5.1	1.1	<b>0.2</b>	0.3	0.6	0.5	0.6	0.6	0.7	0.8	0.7	0.8
V. u. rakosiensis	5.0	4.6	4.9	5.0	4.9	5.2	5.0	4.8	4.7	5.0	5.0	5.2	5.1	1.2	1.1	<b>0.1</b>	0.6	0.6	0.6	0.6	0.7	0.8	0.7	0.8
V. u. ursinii	4.0	3.8	4.0	4.0	3.7	4.1	4.1	3.7	3.8	4.2	4.0	4.7	4.4	3.6	3.8	4.0	<b>0.4</b>	0.4	0.5	0.6	0.6	0.7	0.7	0.8
V. u. ssp.	3.8	3.8	3.9	3.9	3.7	3.8	3.9	3.5	3.8	4.0	3.9	4.7	4.3	3.5	3.8	4.0	2.7	<b>0.3</b>	0.5	0.6	0.6	0.7	0.7	0.8
V. u. graeca	4.2	4.0	4.4	4.2	4.3	4.7	4.4	4.3	4.3	4.7	4.3	4.7	4.8	4.4	4.8	4.7	4.0	3.8	<b>0.9</b>	0.6	0.6	0.7	0.7	0.7
V. kaznakovi Russia	3.4	3.7	3.9	4.0	3.7	4.0	4.0	3.5	3.6	4.1	4.0	4.5	4.5	4.4	4.7	4.7	4.2	4.2	4.4	<b>0.2</b>	0.6	0.7	0.7	0.7
V. kaznakovi Georgia, Turkey	6.5	5.8	6.4	5.8	5.9	6.1	6.5	5.7	5.7	6.0	6.0	6.4	6.0	5.7	6.2	5.8	5.5	5.4	5.6	5.3	<b>0.8</b>	0.7	0.7	0.7
V. anatolica	5.8	5.7	5.8	5.7	5.7	6.0	5.9	5.6	5.9	6.0	5.9	6.4	6.3	6.0	6.2	6.5	5.2	5.1	6.1	5.4	5.9	<b>n/c</b>	0.7	0.8
V. berus	5.7	5.4	5.8	5.4	5.7	5.8	6.0	5.4	5.3	5.7	5.8	6.2	5.7	5.8	6.0	6.2	5.8	5.7	5.9	5.9	5.8	5.9	<b>n/c</b>	0.7
V. seoanei	6.8	6.3	7.0	6.3	6.8	7.1	7.2	6.6	6.8	7.0	7.0	7.3	6.8	6.8	7.4	7.2	7.0	7.1	6.4	6.7	6.8	6.8	4.9	<b>n/c</b>
	Krasnodar 1 (K1), V. r. renardi (individual clades)	West V. r. renardi (WR)	V. r. renardi N-W Caucasus (NWC)	Crimea (RC)	Central N Caucasus (CNC)	V. lotievi Ingushetia (LI)	ast V. r. renardi (ER)	V. lotievi Dagestan (LD)	V. lotievi Chechnya (LC)	V. r. tienshanica W (WT)	V. r. tienshanica (ET)	V. erivanensis	V. ebneri	V. u. macrops	V. u. ursinii	V. kaznakovi Russia	V. kaznakovi Georgia, Turkey	V. anatolica	V. berus	V. seoanei				
<b>Panel B</b>																								
Krasnodar 1 (K1), V. r. renardi (individual clades)	<b>n/c</b>	0.2	0.2	0.2	0.2	0.2	0.3	0.3	0.3	0.4	0.3	0.4	0.5	0.6	0.6	0.6	0.7	0.7	0.7	0.8				
West V. r. renardi (WR)	0.3	<b>0.1</b>	0.2	0.3	0.2	0.3	0.3	0.3	0.4	0.4	0.4	0.4	0.6	0.6	0.6	0.6	0.7	0.7	0.8	0.8				
V. r. renardi N-W Caucasus (NWC)	0.2	0.5	<b>n/c</b>	0.3	0.2	0.3	0.3	0.3	0.4	0.4	0.3	0.5	0.6	0.6	0.6	0.6	0.7	0.7	0.8	0.8				
Crimea (RC)	0.6	0.8	0.7	<b>0.6</b>	0.2	0.3	0.3	0.3	0.4	0.4	0.3	0.4	0.5	0.6	0.6	0.6	0.7	0.7	0.7	0.8				
Central N Caucasus (CNC)	0.4	0.7	0.6	0.8	<b>0.3</b>	0.2	0.3	0.3	0.3	0.4	0.3	0.4	0.5	0.6	0.6	0.6	0.7	0.7	0.8	0.8				
V. lotievi Ingushetia (LI)	0.5	0.8	0.8	1.1	0.7	<b>0.2</b>	0.3	0.4	0.3	0.4	0.4	0.5	0.6	0.6	0.6	0.6	0.7	0.7	0.8	0.8				
East V. r. renardi (ER)	0.7	0.9	0.9	1.0	0.8	1.0	<b>1.0</b>	0.3	0.3	0.4	0.4	0.4	0.5	0.6	0.6	0.6	0.7	0.7	0.7	0.8				
V. lotievi Dagestan (LD)	0.8	1.1	1.0	1.4	1.2	1.3	1.2	<b>0.2</b>	0.4	0.4	0.3	0.4	0.5	0.6	0.6	0.6	0.7	0.7	0.8	0.8				
V. lotievi Chechnya (LC)	1.1	1.4	1.3	1.7	1.1	1.2	1.2	1.4	<b>n/c</b>	0.4	0.4	0.5	0.6	0.6	0.6	0.6	0.6	0.7	0.7	0.8				
V. r. tienshanica W (WT)	1.8	2.0	2.0	2.3	1.9	2.2	2.0	2.1	1.9	<b>1.3</b>	0.3	0.5	0.6	0.7	0.7	0.6	0.7	0.7	0.8	0.8				
V. r. tienshanica (ET)	1.2	1.5	1.2	1.5	1.4	1.6	1.7	1.5	1.7	1.7	<b>1.0</b>	0.4	0.6	0.7	0.7	0.6	0.7	0.8	0.8	1.0				
V. erivanensis	1.7	2.0	2.0	2.0	2.1	2.2	2.0	1.7	2.6	3.0	2.1	<b>0.1</b>	0.4	0.6	0.6	0.6	0.5	0.6	0.6	0.7	0.7	0.8	0.8	
V. ebneri	2.5	2.8	2.8	2.7	2.9	3.1	2.6	2.5	3.1	3.7	3.0	1.3	<b>n/c</b>	0.7	0.6	0.6	0.7	0.8	0.8	0.8	0.8	0.8	0.8	
V. u. macrops	4.2	4.4	4.4	4.6	4.5	4.4	4.6	4.4	4.6	5.3	4.5	4.0	4.8	<b>n/c</b>	0.7	0.5	0.6	0.7	0.7	0.8	0.8	0.8	0.8	
V. u. ursinii	4.2	4.4	4.4	4.6	4.2	4.6	4.5	4.2	4.5	5.0	4.4	4.2	4.8	3.2	<b>n/c</b>	0.6	0.7	0.7	0.8	0.8	0.8	0.8		
V. kaznakovi Russia	3.6	3.9	3.8	4.0	3.9	4.1	4.1	3.7	4.0	4.5	3.9	3.4	4.1	2.9	3.5	<b>0.3</b>	0.6	0.7	0.7	0.8	0.8	0.8		
V. kaznakovi Georgia, Turkey	4.7	4.8	4.8	4.8	4.8	5.2	5.0	4.5	5.1	5.6	4.8	4.3	5.3	5.1	5.9	4.4	<b>0.4</b>	0.7	0.7	0.8	0.8	0.8		
V. anatolica	5.3	5.6	5.5	5.6	5.5	5.8	5.5	5.3	5.6	6.3	5.8	5.5	6.1	5.5	5.9	5.4	5.9	<b>n/c</b>	0.8	0.9	0.9	0.9		
V. berus	6.3	6.4	6.3	6.2	6.6	6.6	6.5	6.1	6.5	7.0	5.9	6.0	6.6	6.2	6.6	5.8	6.3	7.3	<b>n/c</b>	0.8	0.8	0.8		
V. seoanei	6.6	6.6	6.8	6.6	6.9	7.2	6.8	6.8	7.2	7.1	7.4	6.7	6.8	6.7	7.3	6.7	7.0	6.9	5.9	<b>n/c</b>	0.8	0.8		



**Fig. 1.** Maximum Parsimony (MP), Bayesian Inference (BI) and Maximum Likelihood phylogenies reconstructed using two mt-genes (Cytb, COI) with 2021 alignments. The maximum Parsimony tree was calculated using PAUP\*4.0b10 (Swofford, 2001), the Bayesian 50% majority-rule consensus tree using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) and the maximum likelihood tree constructed in PhyML 3.0 (Guindon and Gascuel, 2003). Names of haplogroups are underlined.



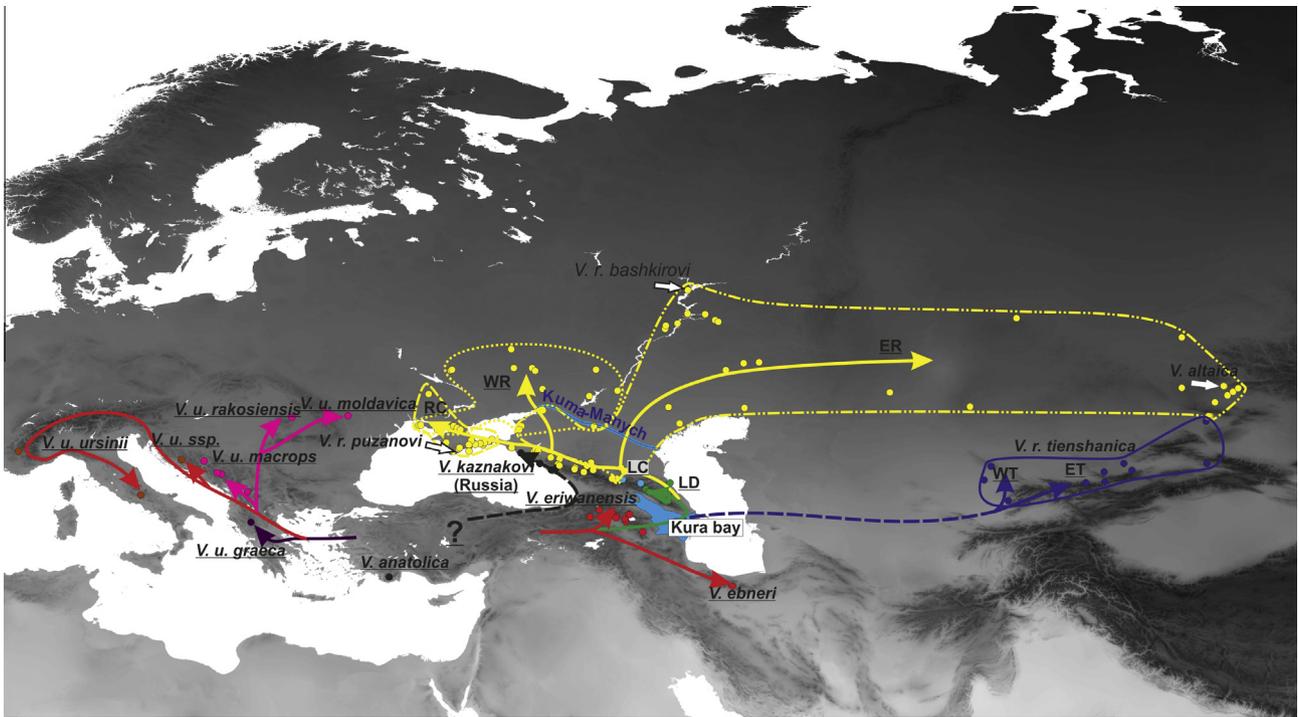
**Fig. 2.** Maximum credibility chronogram (MCC) inferred using Bayesian molecular clocks in BEAST v.1.7.5 (Drummond and Rambaut, 2007) with bars, showing the 95% HPD of divergence time estimates. The phylogram was inferred from two mt-genes (Cytb, COI) with 2021 alignment positions under the best fit model (GTR + I + G).

and represented by a high number of haplogroups with unresolved relationships between them.

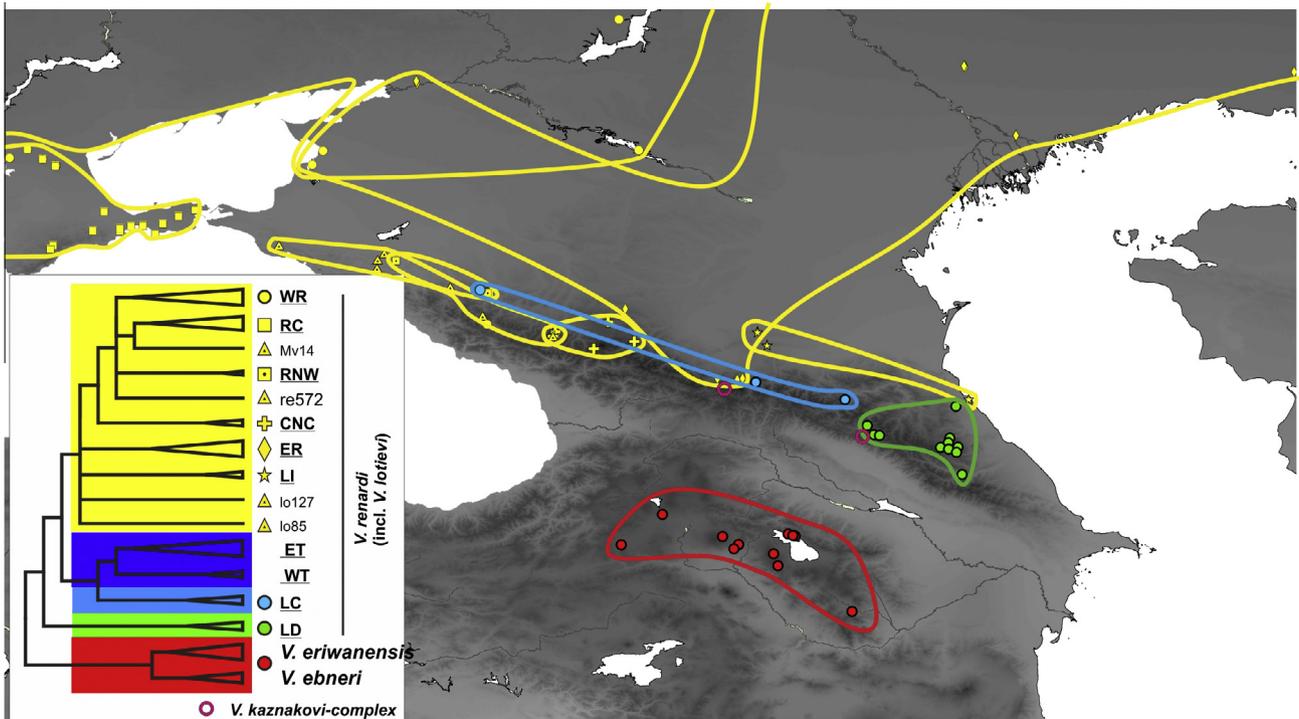
*V. r. tienshanica* from Central Asia is forming a monophyletic lineage (37%/0.98) with two highly supported sublineages: “*V. r. tienshanica* E” (ET – northern Kyrgyzstan to south-eastern Kazakhstan, northern-eastern Tien-Shan Mountains) and “*V. r. tienshanica* W” (WT – Western Tien-Shan Mountains (including the Karatau Ridge in Kazakhstan and Uzbekistan) (Fig. 3). These lineages had a considerable *p*-distance between them (Table 2). A single sequence of *V. r. parursinii* from GenBank (Ferchaud et al., 2012) nested inside *V. r. tienshanica* haplotype ET in spite of a unique combination of morphological peculiarities of this taxon according to description (Nilson and Andrén, 2001). Sample of this taxon was

taken from the territory where only *V. r. parursinii* occurs (Ferchaud, personal communication).

*V. r. renardi* subclade consists of a number of haplogroups. The haplotypes of *V. altaica* and *V. r. bashkirovi* nested inside monophyletic “East renardi” (ER) and shared among a number of populations distributed in the vast lowland steppe areas of Russia from Ciscaucasia and the Volga River to the easternmost part of the range of *V. renardi* (Figs. 1 and 3). Samples of recently described *V. r. puzanovi* from the Crimean mountains shared a haplotype with the lowland Crimean and Ukrainian mainland populations of *V. r. renardi* from the right bank of the Dnieper River near Kirovograd (RC; Fig. 3). Several geographically restricted lineages occurred along the northern macroslope of the Great Caucasus (Fig. 4): **LI**



**Fig. 3.** Distribution of haplotypes of the *ursinii-renardi* group. White arrows indicate *terra typica* of some recently described taxa. Colonization routes are shown with colored lines. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



**Fig. 4.** Distribution of haplotypes of the *V. renardi* group in the Caucasus region. Localities of some specimens misidentified as *V. lotievi* representatives of the *V. kaznakovi* complex (Zinenko et al., unpublished) are shown.

– “Lotievi Ingushetia”, three haplotypes of *V. lotievi* from Tersky and Sunzhensky ridges, northern Ingushetia, and one specimen from Izberbasha in lowland Dagestan; **CNC** – “North-central Caucasus”, three haplotypes morphologically assigned to *V. dinniki* and *V. lotievi* from Karachay-Cherkessia and Kabardino-Balkaria; **RNW** – “*V. r. renardi*, North-West Caucasus”, two haplotypes from the central part of Krasnodar region; and four individual

haplotypes, representing *V. r. renardi* and *V. lotievi* from an area between the central part of Krasnodar region in the West and Karachay-Cherkessia in the East.

Despite ranging from North-Western China and Central Asia to Central Ukraine, and a large number of haplotypes, *V. renardi* was characterized by a shallow phylogenetic structure and a weak topological resolution of mitochondrial phylogenetic tree (Fig. 1).

Genetic *p*-distances between haplotypes within *renardi*-clade were below 2% and rarely exceeded 0.5% within terminal clades (Table 2), indicating a relatively younger and faster radiation compared with the *ursinii* clade. Bootstrap values of many sublineages of *V. r. renardi* in the ML tree were very low, in spite of high posterior probabilities in the BI tree.

### 3.2. Estimated divergence times

Both analyses with entire dataset and coalescence tree model and restricted dataset and Yule tree model has highly congruent topology and close estimation of event date estimation, however timing of deeper branches is slightly bigger and in a terminal nodes slightly less inside *ursinii* complex and *renardi* complex. Results of the molecular datings are given in Fig. 2 as chronograms with 95% confidence intervals. Divergence dates and the time to the MRCA ( $t_{\text{mrca}}$ ) are given in Table 3.

Subgenus *Pelias* had a Late Miocene origin (95% HPD 5–11 Mya). The *ursinii-renardi* clade (including haplotypes of *V. kaznakovi* from

the Russian Caucasus) split from the sister clade of Turkish *V. kaznakovi* at the Miocene-Pliocene boundary. The divergence of *renardi* and *ursinii* complexes was the oldest split inside this group (3.92–4.02 Mya, Pliocene). Russian *V. kaznakovi* and *V. u. graeca* split from *V. ursinii* in Pliocene and subsequent cladogenesis in the *ursinii* complex occurred from the second half of the Pliocene to the Pliocene–Pleistocene boundary, including the separations of *V. u. ursinii*, *V. u. ssp.*, and the Balkan clade of *V. ursinii* (*V. u. macrops* + *V. u. rakosiensis* + *V. u. moldavica*). Early-middle Pleistocene splits inside the *renardi* complex were estimated for *V. eriwanensis*, *V. ebneri*, *V. lotievi* from Dagestan and Chechnya, *V. r. tienshanica*. The same timing applied to splits in the *ursinii* complex inside the Balkan clade, including *V. u. macrops*, *V. u. rakosiensis*, and *V. u. moldavica* (Table 3).

The  $T_{\text{MRCA}}$  for smaller, geographically restricted lineages varied (Table 3). Densely sampled populations of *V. renardi* from lowlands and the North Caucasus (excluding *V. r. tienshanica* and *V. r. parursinii*) showed a tendency for decreasing branching time from 1.06 to 0.45 Mya when moving from East to West.

**Table 3**

Results of Bayesian coalescent-based estimation of divergence dates and the time to the most recent ancestor ( $t_{\text{mrca}}$ ) of extant haplotypes of different monophyletic groups (Fig. 1) calculated using all sequences and coalescent tree model and 23 sequences (one sequence for each monophyletic allopatric group), Yule tree model. Median values in bold and 95 HPD in brackets. See Fig. 1 and Supplementary Table 2 for group composition and names.

Split or population/population	Divergence time (Mya), coalescent tree model	Divergence time (Mya), Yule tree model
<i>Pelias</i> vs. <i>V. monticola</i>	<b>12.16</b> (8.41–17.04)	<b>12.65</b> (8.66–18.05)
<i>berus-seoanei</i> vs. <i>kaznakovi-ursinii-renardi</i>	<b>7.21</b> (5.29–9.81)	<b>7.63</b> (5.32–10.85)
<i>berus</i> vs. <i>seoanei</i>	<b>5.67</b> (3.53–8.17)	<b>6.08</b> (3.74–9.05)
<i>anatolica</i> vs. <i>kaznakovi-ursinii-renardi</i>	<b>5.28</b> (3.88–6.95)	<b>5.55</b> (3.93–7.7)
<i>kaznakovi</i> Turkey vs. <i>kaznakovi</i> Russia- <i>ursinii-renardi</i>	<b>4.99</b> (3.64–6.48)	<b>5.21</b> (3.77–7.31)
<i>ursinii-kaznakovi</i> Russia vs. <i>renardi</i>	<b>3.92</b> (2.93–5.15)	<b>4.04</b> (2.83–5.63)
<i>kaznakovi</i> Russia vs. <i>ursinii</i>	<b>3.20</b> (2.38–4.26)	<b>3.37</b> (2.26–4.81)
<i>graeca</i> vs. <i>ssp.-ursinii-macrops-rakosiensis-moldavica</i>	<b>3.48</b> (2.57–4.62)	
<i>ssp.-ursinii</i> vs. <i>macrops-rakosiensis-moldavica</i>	<b>2.46</b> (1.72–3.29)	<b>2.4</b> (1.42–3.65)
<i>ssp.</i> vs. <i>ursinii</i>	<b>2.03</b> (1.41–2.93)	
<i>rakosiensis-moldavica</i> vs. <i>macrops</i>	<b>0.89</b> (0.53–1.36)	
<i>rakosiensis</i> vs. <i>moldavica</i>	<b>0.65</b> (0.38–1.01)	
<i>eriwanensis-ebneri</i> vs. <i>renardi</i>	<b>1.95</b> (1.38–2.66)	<b>1.93</b> (1.24–2.81)
<i>V. eriwanensis</i> – <i>V. ebneri</i>	<b>1.03</b> (0.67–1.50)	<b>0.87</b> (0.41–1.47)
<i>V. r. tienshanica</i> – the rest of <i>V. renardi</i>	<b>1.26</b> (0.89–1.70)	<b>1.18</b> (0.72–1.74)
<i>V. r. tienshanica</i> West Tien-Shan ( <b>WT</b> ) – <i>V. r. tienshanica</i> East Tien-Shan ( <b>ET</b> )	<b>1.09</b> (0.75–1.49)	<b>0.94</b> (0.53–1.44)
<i>V. lotievi</i> Chechnya ( <b>LC</b> ) – ( <i>V. lotievi</i> Dagestan and the rest of <i>V. renardi</i> )	<b>1.06</b> (0.74–1.45)	
<i>V. lotievi</i> Dagestan ( <b>LD</b> ) – the rest of <i>V. renardi</i>	<b>0.96</b> (0.67–1.33)	<b>0.97</b> (0.58–1.47)
East <i>V. renardi</i> ( <b>ER</b> ) – the rest of <i>V. renardi</i>	<b>0.77</b> (0.52–1.08)	
<i>V. lotievi</i> Ingushetia ( <b>LI</b> ) – the rest of <i>V. renardi</i>	<b>0.67</b> (0.45–0.94)	
<i>V. renardi</i> Central N Caucasus ( <b>CNC</b> ) – the rest of <i>V. renardi</i>	<b>0.57</b> (0.36–0.8)	
<b>Krasnodar 2</b> ( <b>K2</b> ), <i>V. r. renardi</i> – the rest of <i>V. renardi</i>	<b>0.47</b> (0.30–0.67)	
<i>V. renardi</i> NW Caucasus ( <b>NWC</b> ) – <i>V. renardi</i> Crimea ( <b>RC</b> )	<b>0.38</b> (0.23–0.55)	
West <i>V. renardi</i> ( <b>WR</b> ) – rest of <i>V. renardi</i> N-W Caucasus	<b>0.30</b> (0.15–0.49)	
	$t_{\text{mrca}}$	
<i>V. u. graeca</i>	<b>0.88</b> (0.41–1.55)	
<i>V. u. ssp.</i>	<b>0.47</b> (0.20–0.86)	
<i>V. u. ursinii</i>	<b>0.40</b> (0.19–0.67)	
<i>V. u. macrops</i>	<b>0.27</b> (0.08–0.58)	
<i>V. u. moldavica</i>	<b>0.25</b> (0.11–0.45)	
<i>V. u. rakosiensis</i>	<b>0.21</b> (0.09–0.38)	
<i>V. eriwanensis</i>	<b>0.52</b> (0.27–0.84)	
<i>V. ebneri</i>	<b>0.64</b> (0.33–1.03)	
<i>V. r. tienshanica</i> West ( <b>WT</b> )	<b>0.79</b> (0.50–1.14)	
<i>V. r. tienshanica</i> East ( <b>ET</b> )	<b>0.72</b> (0.40–1.10)	
<i>V. lotievi</i> Chechnya ( <b>LC</b> )	<b>0.37</b> (0.10–0.78)	
<i>V. lotievi</i> Dagestan ( <b>LD</b> )	<b>0.28</b> (0.1–0.52)	
East <i>V. renardi</i> ( <b>ER</b> )	<b>0.40</b> (0.22–0.63)	
<i>V. lotievi</i> Ingushetia ( <b>LI</b> )	<b>0.22</b> (0.08–0.42)	
<i>V. renardi</i> Central N Caucasus ( <b>CNC</b> )	<b>0.23</b> (0.09–0.41)	
<i>V. renardi</i> Crimea ( <b>RC</b> )	<b>0.23</b> (0.11–0.37)	
<i>V. renardi</i> NW Caucasus ( <b>NWC</b> )	<b>0.14</b> (0.01–0.32)	
West <i>V. renardi</i> ( <b>WR</b> )	<b>0.22</b> (0.09–0.37)	

### 3.3. Historical demography

#### 3.3.1. The ursinii complex

Mismatch analyses and summary statistics (Table 4) of the entire *ursinii* complex (Fig. 5d) and subsets from Balkan *V. u. rakosiensis*, *V. u. moldavica* and *V. u. macrops* (Fig. 5e) could not reject the null hypothesis of a constant population size. The same result was obtained for geographically restricted *V. u. ursinii* and *V. u. ssp.* (Fig. 5f).

A Bayesian skyline plot of the *ursinii* complex (Fig. 6) suggested an intricate demographical history. This discovery agreed with the results of the mismatch distribution analysis and summary statistics of genetic diversity. The population size might have decreased gradually reaching a minimum about 90 Kya and subsequently, rapid growth had occurred.

#### 3.3.2. The renardi complex

The *renardi* complex had two vicariant clusters: a geographically restricted southern lineages (*V. eriwanensis* and *V. ebneri*) and a northern lineages from north of the main ridge of the Caucasus (*V. renardi*, *V. lotievi*). In contrast to the *ursinii* complex, all non-coalescent and coalescent based methods rejected the null hypothesis of population stability, both for the complete *renardi* complex as well as its southern and northern lineages separately (Table 4). Pairwise mismatch distributions tended to be unimodal, especially those for the *renardi*-clade without *V. eriwanensis* and *V. ebneri* (Fig. 5b). This fits the expectation for sudden population growth. Summary statistics (Table 4) confirmed the mismatch analysis. In all cases,  $F_s$ ,  $R_2$ ,  $r$  and Tajima's  $D$  significantly rejected the null hypothesis of constant population size and revealed evidence of sudden demographic expansion both for the entire *renardi* complex as well as both its' lineages separately.

Bayesian skyline plots visualised changes in the effective population size through time. They revealed a two-stage increase in the effective population size of the *renardi* complex during the Pleistocene (Fig. 6). The effective population size of the entire *renardi* complex gradually increased between 1.25 and 0.5 Mya. Following a period of apparent stability (0.5–0.20 Mya), a second population expansion occurred between 190 and 70 Kya after the differentiation of the localized lineages. After attaining a maximum effective

population size during the Last Glaciation, the population size tended to be stable from 50 Kya until now.

## 4. Discussion

### 4.1. Evolution and biogeography of the ursinii-renardi group

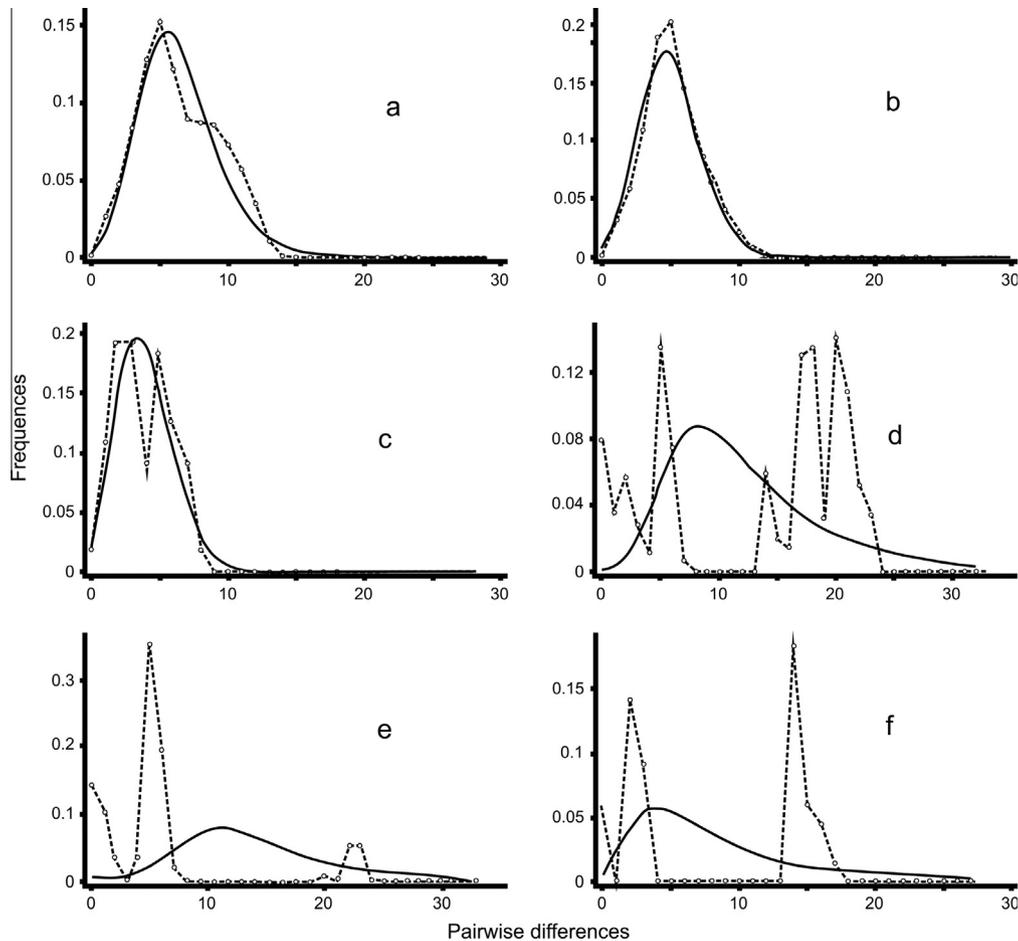
#### 4.1.1. The volume of ursinii-renardi group and relationships between major lineages of Pelias

Unlike Ferchaud et al. (2012), we incorporated in our dataset sequences of *V. kaznakovi* considered as a sister clade to *ursinii-renardi* group (Joger et al., 2007; Gvozdik et al., 2012; Ferchaud et al., 2012) and *V. anatolica*, believed to be a representative of the *ursinii-renardi* group (Nilson and Andr n, 2001; Ferchaud et al., 2012). Our reconstructions placed *V. anatolica* outside of the entire clade of *ursinii-renardi* together with all lineages within *kaznakovi* complex. *V. u. graeca* and one of two lineages of *kaznakovi* complex (haplogroup sampled in *V. orlovi* and *V. kaznakovi* from the Russian part of the Western Caucasus) appeared to be separate lineages near the root of the *ursinii* complex but clearly inside *ursinii-renardi* group. These results are contradicting the current views on a phylogeny of subgenus *Pelias*. There is no significant gap in the distribution as well as any strong morphological or ecological differences between two *V. kaznakovi* lineages, identified here. In the same time, the traditional placing of *V. anatolica* inside the *ursinii-renardi* group has much more sense, because it shares with all species in it similar habitats and situated between the ranges of them; it has much less similar features with *V. kaznakovi* (Nilson and Andr n, 2001). So, we do not include *V. kaznakovi* haplogroup from Russia to the *ursinii-renardi* group. Relationships between *V. ursinii*, *V. u. graeca*, *V. anatolica* and different lineages of *V. kaznakovi* need to be further studied, perhaps with more loci and other approaches to reconstruction of species tree, instead of relying on a single mitochondrial locus, which seems to be misleading in reconstructions of deep divergence of lineages with contrasting demographic history. In the same time, our results agree with the previous studies (Ferchaud et al., 2012; Gvozdik et al., 2012) in the highly supported monophyly of the both *ursinii-renardi* group and *ursinii* (except of *V. u. graeca*) and *renardi* clades and this result seems robust irrespective to sample number, loci and inclusion of other lineages to the analyses.

**Table 4**

Summary statistics and test of population expansion for different subsets of *ursinii-renardi* group. We run separate analyses for the *renardi* complex and northern subclade of *renardi* complex (*V. renardi*, *V. lotievi*) using the concatenated alignment of *Cytb* and *COI* while excluding some short sequences; this reduced the number of haplotypes. Positions with gaps were excluded from analysis and give final length of concatenated sequence. To enlarge the number of sequences used in analysis, we trimmed the sequences to 540 bp fragment present in all sequences, including those that were retrieved from GenBank. Sequence length (L), number of samples (n), haplotype diversity (Hd), nucleotide diversity  $\Pi$  ( $\pi$ ), mean number of pairwise nucleotide differences (k), Fu's statistics ( $F_s$ ), Ramos-Onsins and Rozas's statistics ( $R_2$ ), Harpending's raggedness index statistics ( $r$ ), and Tajima's  $D$  (D) are given. Fu's  $F_s$ , Ramos-Onsins and Rozas's  $R_2$  and the raggedness index were estimated based on the coalescent simulations with 10,000 replicates in consideration of no recombination. Statistics significantly (all  $p$  values < 0.05) differ from expectations based on the null model of neutral evolution in population of constant size (in bold).

Population	Locus	Length	n	Hd	k	Pi	Fs	R <sub>2</sub>	r	D
<i>eriwanensis-ebneri-renardi</i> (Cytb + COI)	Cytb, COI	1848	26	1	24.409	0.0321	-10.193	0.0678	0.0052	-1.52735
<b>p</b>							<b>0.0017</b>	<b>0.0097</b>	<b>0.0085</b>	<b>0.042</b>
<i>renardi</i>	Cytb, COI	1848	23	1	20.458	0.01107	-9.363	0.07	0.0063	-1.50931
<b>p</b>							<b>0.0029</b>	<b>0.0097</b>	<b>0.0089</b>	<b>0.046</b>
<i>eriwanensis-ebneri-renardi</i> (Cytb)	Cytb	540	56	0.993	6.250	0.01157	-69.7518	0.0462	0.0077	-1.77976
<b>p</b>							<b>0.000001</b>	<b>0.015</b>	<b>0.04705</b>	<b>0.003</b>
<i>renardi</i>	Cytb	540	47	0.991	4.950	0.00917	-61.525	0.0416	0.0176	-2.00891
<b>p</b>							<b>0.000001</b>	<b>0.0033</b>	0.0593	<b>0.0059</b>
<i>eriwanensis-ebneri</i>	Cytb	540	11	0.964	3.818	0.00707	-3.713	0.1261	0.0555	-0.29553
<b>p</b>							<b>0.01471</b>	0.12277	0.15253	0.41520
<i>ursinii-clade</i> (without <i>graeca</i> ) (Cytb)	Cytb	540	15	0.9312	13.011	0.02409	1.086	0.134226	0.0538	0.3066
<b>p</b>							0.6930	0.741	0.904	0.688
<i>macrops-rakosiensis-moldavica</i>	Cytb	540	7	0.843	5.856	0.01084	1.865	0.1682	0.1741	-1.1223
<b>p</b>							0.8193	0.8297	0.9188	0.1262
<i>ursinii, ssp., (Alps, Apennines, partly Balkans)</i>	Cytb	540	8	0.897	8.436	0.01562	0.6922	0.1732	0.234057	0.8179
<b>p</b>							0.6281	0.7751	0.96	0.8378
Entire dataset	Cytb	540	70	0.989	13.667	0.02533	-34.093	0.0783	0.0062	-0.75692
<b>p</b>							<b>0.000001</b>	0.32940	<b>0.0498</b>	0.2519



**Fig. 5.** Mismatch distributions for different subsets of the *ursinii-renardi* group. Solid lines indicate the observed frequency of pairwise nucleotide differences between sequences, and dashed lines represent the expected distribution based on a model of sudden population growth. To enlarge the number of sequences used for analysis, we trimmed the sequences to a 540 bp fragment present in all sequences, including those that were retrieved from GenBank. (a) *renardi* complex, 56 haplotypes; (b) northern subclade of *renardi* complex (*V. renardi*, *V. lotievi*) 48 haplotypes; (c) southern subclade of *renardi* complex (*V. ebneri*, *V. eriwanensis*) 10 haplotypes; (d) *ursinii* complex (without *V. u. graeca*), 15 haplotypes; (e) *V. u. macrops*, *V. u. rakosiensis*, *V. u. moldavica*, 19 haplotypes; (f) *V. u. ursinii*, *V. u. ssp.* (Alps and Apennines), 16 haplotypes.

#### 4.1.2. Origin of the group, dispersion route and timing of main cladogenesis

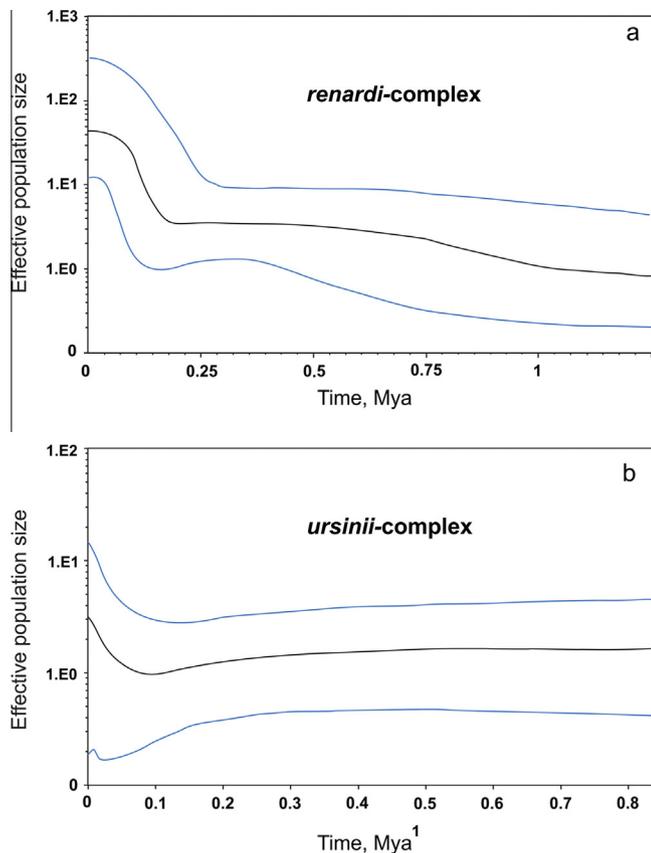
Our estimations deviated systematically from the analogous results of Ferchaud et al. (2012) in tending to be older at the base of the tree and younger in the terminals. Explanation of this difference could be in using different calibration points and tree model. We believe that using biogeographically validated calibration points inside Old World vipers, not Crotalids as in Ferchaud et al. (2012) is the best currently available alternative we had.

The initial distribution of the common ancestors of subgenus *Pelias* could be associated with Late Miocene European land to the North from Paratethys and stretching from Iberian peninsula in the west to the Caucasus in the East (Starobogatov, 1994; Popov et al., 2004; Krijgsman et al., 2010). The initial split of *Pelias* on two groups with hypothesized centres of origin in Europe (*V. seoanei* and *V. berus*) and Asia (*ursinii-renardi* group and *V. kaznakovi* complex) 7.21 Mya according to our timing (Table 3) may relate to the intensive orogenesis in Europe and Asia Minor (Velichko and Spasskaya, 2002) and subsequent transgressions of the Paratethys about 8.5–7 Mya (Popov et al., 2004), which isolated these two regions by forming a strait between Eastern Mediterranean and Black sea basins. The split of the *ursinii-renardi* group and the *V. kaznakovi* complex (*V. kaznakovi* of Georgia and Turkey) dates back to the Pliocene, about 5 Mya and could be associated with Zanclean flood and transgression of the Eastern Paratethys Sea after the end of Messinian crisis (Popov et al., 2004) which have led to an

temporary isolation of the Caucasus from Asia Minor. We restrict hypothetical place of origin of the *ursinii-renardi* earlier identified as the territory between the Balkans and the Caucasus by Ferchaud et al. (2012) to Asia Minor.

The Pliocene climatic optimum 4.2–4.0 Mya could have triggered the altitude shift and adaptation to open mountainous grassland habitats in the ancestor of the *ursinii-renardi* group. This adaptation allowed later the ancestors of *V. ursinii* to enter Europe using the inland connection between the Balkans and Asia Minor established during one of Paratethys regressions (Starobogatov, 1994) and first colonize mountainous habitats in Southern Balkans. Split between *ursinii* complex and *renardi* complex around 4 Mya coincides in time with estimation of the split between Balkan and Turkish lineages of green frogs of the genus *Pelophylax* (Lymberakis et al., 2007), indicating on same paleogeographic event, probably transgression, which resulted in vicarious evolution in the region.

Further dispersion of *ursinii* and *renardi* clades should occur in the north-west and north-east directions. The *ursinii* complex spread across the Balkans from the Pindos Mountains in Greece (*V. u. graeca*) via the Dinaric Alps (*V. u. macrops* and *V. u. ssp.* from Bosnia and Herzegovina and Croatia) and finally to the French Alps and the Apennines (*V. u. ursinii*). Lowland *V. u. moldavica* and *V. u. rakosiensis* split from the common stem with *V. u. macrops* and dispersed north-eastwards. The eastern *renardi* lineage dispersed north-eastwards via Armenian upland (*V. eriwanensis*), reaching



**Fig. 6.** Demographic history (effective population size) of the *renardi* complex (a) and the *ursinii* complex without *V. u. graeca* haplotypes (b) as a function of time (Mya), determined with BEAST v1.7.5 (Drummond and Rambaut, 2007) Bayesian skyline plot using the GTR + G + I evolution model for 300 million generations. The central line indicates the median value for the effective population size ( $N_e$ ) on a logarithmic scale and the blue lines outline the 95% highest posterior density area. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Elburz Range in Iran (*V. ebneri*), Tien-Shan in Central Asia (*V. r. tienshanica* and *V. r. parursinii*), North-east Caucasus (Eastern Caucasus lineages of *V. lotievi* **LD** and **LC**) and via the North and North-west Caucasus to the northern lowland steppes and the Crimea (late Pleistocene lineages of *V. r. renardi*) (Fig. 3). The split between *V. renardi* s. str. and *V. eriwanensis*-*V. ebneri* about 1.96 Mya associates with sea-level fluctuations in Caspian and Black sea basins, especially the post-Akchagyl regression (Van Baak, 2013). This temporary drop of sea level 2.3 Mya followed by a subsequent transgression of Apsheronian and Gury seas around 2 Mya (Starobogatov, 1994; Krijgsman et al., 2010) appears to have allowed the ancestors of *V. renardi* to reach the Eastern Caucasus from Asia Minor via the dry Kura Bay of the Akchagyl Sea (Fig. 3). Presence of *V. eriwanensis*-like snakes in the Northern Azerbaijan, to the south from the main ridge of the Caucasus (Kukushkin et al., 2012) confirms this route of dispersion. The origin of *V. r. tienshanica* (1.27 Mya) appears to reflect further eastern dispersal of steppe vipers via the Elburz, Kopet-Dag and Turkestan ridges and/or the dry Eastern Paratethys Sea towards Tien-Shan. All old lineages of the *V. renardi* complex (*V. lotievi* from East Caucasus; *V. r. tienshanica*) are primarily occupying mountains and we believe that the initial dispersion to the east occurred rather via low mountains, than via lowland steppes. An alternative dispersion way should imply yet another change of preferable habitats by steppe vipers, followed by extinction of the lowland populations. Further splits in the old mountainous lineages of *V. renardi* around 1 Mya indicate an importance of this period for cladogenesis: two

haplogroups of *V. lotievi* from Dagestan and Chechnya split, as did eastern and western haplogroups of *V. r. tienshanica*, as well as *V. eriwanensis* and *V. ebneri*. An early origin of *V. lotievi* (Tuniyev et al., 2011) is suggested because of paleobotanical reconstruction of arid landscapes in Dagestan (Galushko, 1974). The Early Pleistocene, but not Pliocene origin of the species partly confirms this conjecture.

Dense sampling in the North Caucasus shows a prominent diversity of haplotypes there with the radiation falling into the second half of the Pleistocene. We observed a distinct longitudinal localisation and a gradual decrease of genetic differentiation between lineages when moving from the East to the West (Fig. 4, Table 2). The steppes to the north from Caucasus were hypothesized as the place of origin of lowland *V. renardi* (Kukushkin, 2009). This region was isolated from the vast northern steppes with Kuma-Manych strait, periodically connecting the Black Sea and the Caspian Sea basins during transgressions (Starobogatov, 1994; Svitoch, 2008). We believe that this could be the reason why the northward colonization of the lowland steppes across the Kuma-Manych depression occurred comparatively late in the mid Pleistocene and this appears to have involved only two lineages **WR**, **ER**, both still being found in the area to the south from this barrier. First fossils of steppe vipers in the northern steppes (*V. ursinii* s. l., Petropavlovian and Muchkapien horizons of end of Early and beginning of Middle Pleistocene, upper Don basin, Russia – Ratnikov, 2009) are dated to 0.8–0.9 and 0.5–0.6 Mya, close to the time when one of the widespread lowland clades, **ER** already had split off from all other mountain lineages of *V. renardi* (Table 3).

An additional dispersion event had happened when the Crimean *V. renardi* lineage, **RC**, had reached the Crimea via an ephemeral land bridge connected it and the Taman peninsula (Kukushkin, 2009). Alternatively, vipers may have reached these territories around the present Sea of Azov and became isolated about 0.45 (0.27–0.65) Mya.

#### 4.1.3. Transition between high altitude and lowland grasslands

While most of the older lineages of *Pelias* are exclusively or predominantly mountain dwellers, several lineages within *ursinii-renardi* group (*V. u. rakosiensis*, *V. u. moldavica* and *V. r. renardi*) independently developed ability to live in lowland landscapes and were able to colonize vast plain steppes (see also Ferchaud et al., 2012). Apparently an adaptation to mountainous grasslands similar to lowlands steppes in thermal regime and seasonality, habitats type and key prey species served as a first stage in this process. Since we had more data about *V. renardi* we were able to trace this process more in details in the eastern *renardi*-clade. The most widespread lowland haplogroups – western and eastern *V. renardi* (**WR** and **ER**) and Crimean steppe vipers (**CR**) – live on elevations ranging from sea level to over 1000 m a.s.l., however the most of the occupied territories are lowlands. Additionally, several mountain Caucasian haplotypes, including those of *V. lotievi* from Dagestan (**LD**, **LI**, haplotypes from the West Caucasus *V. r. renardi* clade), also were occasionally found in lowland Ciscaucasia (Fig. 4) and are demonstrating altitude plasticity. Thus, we believe that the plasticity in altitude distribution was necessary but not unique in different lineages of the *ursinii-renardi* group and is not sufficient prerequisite of colonization of lowland steppes. The geographic position of initial ranges of haplogroups in relation to suitable dispersal routes best explains their successful dispersion.

Steppe vipers are not unique among vertebrates in their ability to live in both mountain and lowland steppes. Two closely related forms of ground squirrels *Spermophilus musicus* (Menetries 1832) and *Spermophilus pygmaeus* (Pallas, 1778) occur in mountains of Northern Caucasus and northern steppes (Ermakov et al., 2006). The same mitochondrial clade of sand lizard occupies both western

portion of Northern Caucasus and lowland steppes of Southern Russia (Kalyabina-Hauf and Ananjeva, 2004). The corridor for dispersion of steppe biota to the north across the Kuma-Manych depression, or to high altitude habitats on the slopes of North Caucasus appear to be same for different systematic categories and indirectly supports the importance of geographic position of the source population for the successful dispersion. Possibly, at certain period of time in Pleistocene newly emerged lowland populations of steppe vipers between the Caucasus and Kuma-Manych depression could be also a source of colonization of not only northern lowland steppes, but open grasslands along river valleys in the Northern Caucasus. According to Alekseeva and Lomize (1960), the absence of uninterrupted forests in the North Caucasus in the past facilitated the recent invasion of mountains by typical lowland steppes inhabitants: ground squirrels and marmots. In the Middle or Late Pleistocene, steppe vegetation and animals could invade the Caucasus up to the Lagonaky Plateau at approximately 1400–1500 m a.s.l. and went extinct after recovery of forest vegetation. Kidov (2009) suggested the recent invasion of *V. renardi* to Skalisty Ridge (Krasnodar) after discovering a mountain population exhibiting the typical morphology of lowland vipers. During cold, dry glaciation periods, steppe communities could have dispersed upstream along river valleys on northern slopes of the Caucasus. In warm, wet periods populations in the valleys were isolated from the lowland by recovering forests. Thus, most of the young lineages of the North-West and Central Caucasus reflect dead-end expansions of steppe vipers into mountains. This scenario explains the high diversity and heterochrony of radiation of mtDNA haplotypes in the North Caucasus. Morphological diversity there (Tuniyev et al., 2011) likely reflects a localized evolution and in some cases hybridisation with the old lineages of *renardi* complex or even *kaznakovi* complex (Zinenko, Joger, pers. comm.). Therefore we conclude that the North Caucasus and adjacent lowland steppes could served both as hotspot and melting pot for steppe vipers, a phenomenon that also known in Iberian peninsula for *Lacerta schreiberi* Bedriaga, 1878 (Godinho et al., 2008).

#### 4.1.4. Correspondence between demographic data, the colonization events and climate

The biogeographical scenario of the *renardi* complex agrees well with demographic history (Fig. 6) and results of mismatch analysis (Table 4; Fig. 5), however both methods could be sensitive to bottlenecks and therefore could show changes in population size, which had happened only after the last constriction of the range and decrease of population number (Excoffier and Schneider, 1999). Mismatch distribution is also proved to be multimodal in the case of geographic subdivision of populations (Marjoram and Donnelly, 1994) and this is the reason that alternatively explains multimodal distribution and statistical support of constant size of population in different datasets of *V. ursinii* (Table 4; Fig. 5). Effective population size of *renardi* complex has grown after colonization of the Greater Caucasus and Tien-Shan 1.2 Mya. Growth accelerates from 200 Kya, likely after the emergence of several widespread lowland lineages in *V. r. renardi*, the regressions of the Black and Caspian basins, interruption of Kuma-Manych strait (Starobogatov, 1994) and the colonization of lowland steppes. Effective population number growth appears to have continued until the terminal stage of the Last Glacial Maximum both in *ursinii* complex and *renardi* complex (Fig. 6). Late Pleistocene cooling appears to have the rather positive effect on the effective population number of steppe vipers. The reason for that in our opinion lays in the fact that xerophilic communities in southern Eastern Europe and North-West Asia existed both during maximal stages of glaciation as tundra-steppes and during interglacials as forest-steppes (Artyushenko and Turlo, 1989; Yakhimovich et al., 1989; Blagovolin et al., 1982; Markova et al., 2002; Velichko and

Spasskaya, 2002; Simakova, 2008). A success of colonization of a vast lowland steppes and surviving there during climate cooling periods (fossil steppe vipers were found in Bolshye Tigany, Tatarstan, dated to the Last Glaciation and distributed through the plains of Eastern European from the second half of the Middle Pleistocene to the Holocene, encompassing several climatic oscillations – Ratnikov, 2009) is predicted by geliotermity of steppe and meadow vipers and their preferences to occupy open habitats. Being adapted to temperate climate, like most of small vipers of *Pelias* subgenus, steppe and meadow vipers have enough hit coming from sun radiation in open grassland habitats both in mountains of Southern Europe and in dry continental steppes of East Europe and Northern Asia disresponsible to stage of climatic oscillation, which is not a very common situation in reptiles.

In conclusion, our results, as well as those of Ferchaud et al. (2012) and Gvozdik et al. (2012), reject the hypothesis of origin and of the group in the plains of Eastern Europe and Central Asia and further dispersion to mountain habitats and isolation there during the Pleistocene during cold glacial periods (Dely and Stohl, 1989; Nilson and Andrén, 2001) and favour a scenario with the main direction of colonization occurring from south to north and later to east and west. The Turan (Central Asiatic) origin of steppe vipers, postulated by A. M. Nikolsky (1916) is not confirmed. We conclude that the ecological transition from mountain grasslands to lowland steppes had happened independently later in different lineages of the group, which came into contact with steppes of Eastern Europe. Climatic oscillations of Pleistocene had a minor impact on demography of steppe vipers.

#### 4.2. Taxonomic implications

Our results are congruent with the subdivision of the *ursinii-renardi* group into western *V. ursinii* and eastern *V. renardi* complexes. They support taxonomic recognition of species such as *V. eriwanensis*, *V. ebneri* and *V. renardi* (Nilson and Andrén, 2001). The *ursinii* complex contains undescribed *V. u. ssp.* from Croatia, Bosnia and Herzegovina, first discovered by Ferchaud et al. (2012) and also present in Gvozdik et al. (2012) and Stümpel (2012) datasets as *V. u. macrops*. It is the sister lineage of *V. u. ursinii* from Italy and France, which should be studied and gain taxonomic status after formal description.

No evidence suggests independent origins for the recently described *V. altaica* and *V. r. bashkirovi*. The both taxa belong to the ER *V. r. renardi* haplogroup. Morphologically, the both taxa differ from *V. r. renardi* and occur at the edge of the main range of steppe vipers. *V. altaica* does not have the bilineate dorsal pattern typical of most steppe vipers, has a whitish belly, no suture on the supralabials and the highest number of ventrals in the complex in spite of its small size (Nilson and Andrén, 2001; Tuniyev et al., 2010). *V. r. bashkirovi* resembles *V. altaica* in pholidosis, but its larger size constitutes an unusual trait for lowland *V. renardi* and its frequent black body coloration and high level of morphological variation exceeds that within eastern or western *V. renardi* and *V. altaica* (Garanin et al., 2004). At least three explanations exist for the discordance of these morphological peculiarities (Garanin et al., 2004; Ostrovskikh, 2006; Tuniyev et al., 2010) and low genetic distance in mtDNA sequences from lowland ER haplotypes, two of which imply introgression, as listed below.

- (1) Original haplotypes were replaced by introgressive hybridization with *V. r. renardi*, as happens in the Ukrainian and Russian *V. b. berus* and *V. b. nikolskii* (Zinenko et al., unpublished results). MtDNA in general are believed to be prone to asymmetric capture in case of introgression due to direct benefits from the local adaptation and higher probability of drift (e.g. William et al., 2004; Babik et al., 2004; Godinho

et al., 2008). Such mtDNA introgression must have occurred in the past because *V. r. renardi* and *V. r. bashkirovi* are now distributed about 150 km apart (Garanin et al., 2004). The Altai populations also appear to be isolated from *V. renardi* (Tuniyev et al., 2010).

- (2) A rapid adaptive radiation may occur in isolated edge populations and this may result in a discordance between morphological and mtDNA variation. The split between Altai haplotype “alt 10” and other haplotypes of **ER** (the *terra typica* of *V. altaica*) dates to 170 (70–290) Kya. This date conflicts with a Pliocene–Pleistocene age of this taxon (Tuniyev et al., 2010). The same haplotype occurs in *V. r. bashkirovi* (Fig. 1, Supplementary Table 1). Slightly morphologically and ecologically specific lineages of *V. renardi* from the Crimea and the North-west Caucasus (Kukushkin, 2009; Ostrovskikh, 1997, 2006) are at least twice older than those of *V. altaica* (**ER** clade, Table 3). However, several insular populations of *V. renardi* exhibit substantial morphological variation on the background of little observed genetic distance between mtDNA sequences. Giant *V. renardi* from Orlov Island in the Black Sea (Kotenko, 1983) have haplotypes from **WR** and **CR** clades, which occur in mainland populations exhibiting the typical morphology of *V. renardi*. A small isolated population from Velikaya Bagachka, Poltava, Ukraine possesses a high frequency of rare combinations of pholidosis, dark coloration and a unique pattern (Zinenko, unpublished results), yet it possesses the widespread **WR** haplotype of *V. renardi*. In other species, such situation is known in *Vipera aspis* (Linnaeus, 1758), when morphologically different *V. a. aspis* and *V. a. atra* do share the same mtDNA haplotype (Ursenbacher et al., 2006b).
- (3) The other explanation is the hybrid origin of these populations, the both of which occur at the parapatric boundary of *V. renardi* and *V. berus*. Several samples from both taxa are heterozygous for alleles of *V. berus* and *V. renardi* and express intermediate morphologies and venom compositions indicating on hybridization which occurs (Pavlov et al., 2011). One possible hybrid is known from Altai (Zinenko, Stümpel, Duisebayeva, Joger, unpublished results).

Recently described *V. r. puzanovi* possesses a unique haplotype, but in contrast to the restriction of this taxon to the southern Crimea (Kukushkin, 2009), the haplotype occurs across the peninsula and in the adjacent mainland sympatrically with a haplotype of the **WR** haplogroup. The only studied Central Ukrainian population from west of the Dnieper River also belongs to this haplogroup. Therefore the border between *V. r. puzanovi* and *V. r. renardi* in the central Crimea (Kukushkin, 2009) seems incorrect. Further, many diagnostic traits of *V. r. puzanovi* may be epigenetic responses to the environment (see also Kukushkin and Zinenko, 2006).

One surprise involves the relationships of numerous samples from the North Caucasus morphologically assigned to *V. lotievi* and partly to *V. dinniki* Nikol'sky, 1913 (Tuniyev et al., 2009, 2011; Nilson et al., 1995; Kidov et al., 2009). Lotiev's viper is highly polyphyletic and involves two lineages sequentially rooted at the base of the *V. renardi* clade, one from Dagestan and the other from the *terra typica* at the Chechnya–Ingushetia border. Recently, Tuniyev et al. (2011) assigned both lineages to the eastern group of *V. lotievi*. Several more terminal lineages of *V. renardi* determined as *V. lotievi* occur across the North Caucasus westwards of Dagestan (Figs. 1 and 4). Further, some high altitude populations morphologically determined as *V. lotievi* from Dagestan (Tlyarata) and northern Ossetia–Alania (Fiagdon) possess haplotypes of the *V. kaznakovi* species complex (Fig. 4; Zinenko, unpublished results). This pattern may have multiple explanations: (1) morphological

uncertainty in that most diagnoses cannot identify species; (2) morphological convergence among localities may evolve in montane environments (Nilson and Andrén, 2001; Kukushkin, 2009); and (3) hybridization and introgression. Numerous stages of expansion, hybridization and isolation may have driven the admixture of traits. We confirm an on-going hybridization by reporting the discovery of two divergent haplotypes in one locality (Fig. 4) and, better, heterozygotes on nuclear genes (Zinenko, unpublished results).

Mitochondrial DNA sequences lacking ability to evident about gene flow between geographical populations to address the issue of reproductive isolation and the level of genetic differentiation between clades does not necessary corresponds to taxonomic differentiation (Frost and Hillis, 1990). Moreover decision about the taxonomic status depends greatly from species concept and criteria in use. It is difficult to solve all taxonomic problems in *renardi* complex having only these data and further taxonomic rearrangements can only be done on the basis of a large material and integrative approach combining morphological, ecological and genetic multilocus data. Here we prefer to keep a conservative point of view on the taxonomy of the *renardi* complex and refer all populations from Northern Caucasus as *V. renardi* s. l.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ymp.2014.12.005>.

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