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Short Communication

A mitochondrial DNA phylogeny of the endangered vipers of the *Vipera ursinii* complex

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ABSTRACT

The last two populations of the Hungarian meadow viper *Vipera ursinii rakosiensis* were thought to persist in the steppe fragments of Hungary until meadow vipers were discovered in central Romania (Transylvania), suggesting a possible existence of remnant populations elsewhere. We assessed the phylogenetic position of the Transylvanian vipers using 2030 bp of mitochondrial DNA sequence. We showed that they were closely related to the Hungarian vipers, while those from northeastern Romania (Moldavia) and Danube Delta belonged to the subspecies *Vipera ursinii moldavica*. Montane subspecies from Europe (*Vipera ursinii ursinii* and *Vipera ursinii macrops*) formed a sister clade to the two lowland subspecies. *Vipera renardi* formed a sister clade to *V. ursinii*, with populations from the Greater Caucasus (*Vipera renardi lotievi*) and Tien Shan (*Vipera renardi tienshanica*) as the sister group to *Vipera renardi renardi*, and *Vipera renardi eriwanensis* from the Lesser Caucasus as the most basal taxon in the species. Our results illustrate that the divergence between the lowland and montane populations occurred separately in each species and several times in *V. renardi*. We demonstrated that the recently discovered Transylvanian population is the third surviving population of *V. u. rakosiensis* and the only known population outside of Hungary.

1. Introduction

Meadow and steppe vipers of the *Vipera ursinii* species complex are small venomous snakes (40–60 cm in length) specialized to life in grasslands, and their distribution thus encompasses areas of grassland habitats from the south-eastern France through the southern and eastern Europe to Central Asia (Fig. 1). Throughout this vast range, the vipers occupy two principal habitat types: warm dry lowland meadow-steppe grasslands, generally below 400 m a.s.l., and alpine/subalpine (montane) meadow-steppe habitats, typically at altitudes above 1000 m a.s.l. (Nilson and Andrén, 2001). As a result of the habitat specialization, the meadow and steppe vipers have patchy distribution, due in part to the island-like nature of the alpine habitats, but also, especially in Europe, due to an anthropogenic reduction and deterioration of the lowland steppe (Edgar and Bird, 2006). This is particularly true of the Hungarian meadow viper, *Vipera ursinii rakosiensis*, a lowland steppe inhabitant that has disappeared from the most of its native range and presently is considered one of the most critically endangered snakes in Europe (Edgar and Bird, 2006). The historical distribution of *V. u. rakosiensis* extended from the Vienna Basin through the Great Hungarian Plain to Romanian Transylvania (Dely and Joger, 2005; Edgar and Bird, 2006). However, in Romania there were no verified records since the 1950s (Krecsák and Zamfirescu, 2008) and in Austria it disappeared during the 1980s (Grillitsch and Cabela, 2001), so that isolated populations, counting together no more than several hundred individuals, currently survive only in steppe remnants of the north-western (Hanság) and central Hungary (Kiskunság), respectively (Edgar and Bird, 2006; Sós et al., 2006; Újvári et al., 2000; Cheylan et al., 2011). Very small areas of suitable habitat, its fragmented distribution and signs of population inbreeding have led to the suggestion that *V. u. rakosiensis* might be close to extinction (in Hungary and globally) if adequate measures are not taken (Rehák, 2004; Újvári et al., 2000, 2002).

There are other extant populations of *V. ursinii* in Europe, both in lowlands and in alpine habitats. However, the geographically closest lowland populations in northeastern Romania (Moldavia), formerly considered as hybrids of *V. u. rakosiensis* with the eastern

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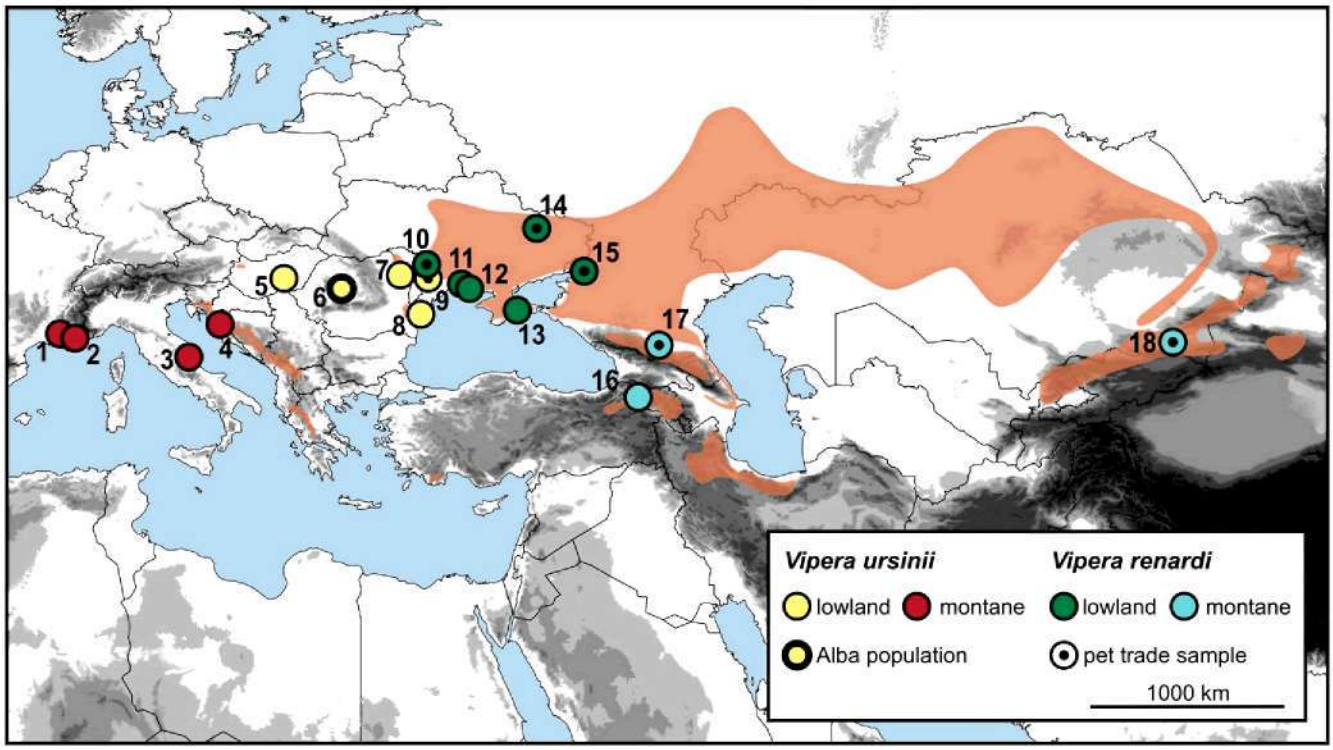

Fig. 1. Geographic origin of the vipers within the distribution range of the *Vipera ursinii* complex (ochre). The range is drawn after Cheylan et al. (2011), David and Vogel (2010), Dely and Joger (2005), Joger and Dely (2005), Korsós et al. (2008), Nilson and Andrén (2001), and Valakos et al. (2008).

Vipera renardi (Fuhn and Vancea, 1961), are now regarded as a distinct subspecies *Vipera ursinii moldavica* (Nilson et al., 1993; Zamfirescu et al., 2009), and vipers from the more southerly Danube Delta may also belong to this subspecies (Dely and Joger, 2005; Nilson and Andrén, 2001). According to some authors, however, vipers from the Danube Delta belong to *V. renardi* (e.g. Fuhn and Vancea, 1961), and their systematic assignment is therefore still unsettled (Speybroeck and Crochet, 2007). *V. renardi* is commonly recognized as a distinct species from *V. ursinii* (Dely and Joger, 2005; Joger and Dely, 2005; Nilson et al., 1995), but some authors consider it a subspecies of *V. ursinii* (e.g. Újvári et al., 2005). The steppe viper *V. renardi* is a widespread species, occurring throughout the steppe regions of Ukraine and southern Russia to Central Asia, with several montane subspecies, including *Vipera renardi lotievi* and *Vipera renardi eriwanensis* in the Caucasus (both often considered full species; e.g. David and Vogel, 2010), and *Vipera renardi tienshanica* in the vicinity of the Tien Shan Mountains (Joger and Dely, 2005). Also the montane populations of *V. ursinii* from Europe are regarded as distinct subspecies from the lowland populations: *Vipera ursinii ursinii* in the Apennines in Italy and in the French Alps (the latter also known as *Vipera ursinii wettsteini*) and *Vipera ursinii macrops* in the Dinaric Alps in north-western Balkans (Dely and Joger, 2005; Nilson and Andrén, 2001). *Vipera ursinii graeca* endemic to the Pindos Mountains in southern Balkans, which is not included in this study, was recently suggested to represent a separate species sister to all other meadow and steppe vipers (Cheylan et al., 2011).

Despite their taxonomic distinctions based on differences in colour pattern (amount of ventral black, shape and size of lateral blotches, colour of margins of labials) and scalation (position of dorsal scale row reductions, number of ventrals), the evolutionary relationships among various lowland and montane populations of *V. ursinii* and *V. renardi* have not been tested using molecular phylogenetic methods. A few *V. ursinii* were included in the adder (*Vipera berus*) phylogeny by Kalyabina-Hauf et al. (2004) but no conclusions were drawn from the limited dataset. For example, it is not clear whether *V. u. rakosiensis* is evolutionarily closest to other lowland taxa (*V. u. moldavica* or *V. renardi*), or if its closest relatives may be found among the montane taxa (Nilson and Andrén, 2001; Nilson et al., 1993). Identification of populations genetically related to the Hungarian populations would be important, for example, if they required genetic augmentation (see Újvári et al., 2002). An important discovery in this respect is the recent finding of a small population of meadow vipers in the lowlands and on the slopes of a hilly area in central-western Romania, in Alba County in Transylvania, at altitudes between 280 and 500 m a.s.l. (Ghira, 2007; Krecsák and Zamfirescu, 2008). Ghira (2007) argued that the morphometry and scalation of vipers from the Alba population were sufficiently similar to data published for *V. u. rakosiensis* to consider them as belonging to this subspecies. However, the Alba population is geographically intermediate between the Hungarian *V. u. rakosiensis* in the west and *V. u. moldavica* and *V. renardi* in the east (including taxonomically disputed vipers from the Danube Delta). Further study is therefore needed to resolve the taxonomic status of this recently discovered population.

Here we use sequences of coding as well as non-coding segments of mitochondrial DNA (mtDNA) to infer the evolutionary relationships among different lowland and montane populations of *V. ursinii* and *V. renardi*. Special focus is given to the relationships of *V. u. rakosiensis* with the other populations and taxa. We for the first time collect DNA sequence data for vipers from the recently discovered Romanian population, which help resolve their systematic position.

2. Material and methods

2.1. Data collection

Oral swabs were used as the source material for DNA extraction with the Macherey–Nagel NucleoSpin kit (Düren, Germany). Field-collected samples were supplemented with sampling of living

captive specimens from the pet trade (wild catches with locality data) and additional sequences were retrieved from GenBank (Table 1). Further included were one *V. berus* and one *Vipera kaznakovi*, which are in the same subgenus (*Pelias*) as the meadow and steppe vipers and served as outgroup (Garrigues et al., 2005). The new mitochondrial DNA sequences encompassed a portion of the 3′ region of the cytochrome *b* gene (*Cytb*), the complete transfer RNA for threonine (*tRNA-Thr*), the complete control region 1 (*CR1*; snakes possess duplicate control region; see e.g. Jiang et al., 2007), the complete tRNA for phenylalanine (*tRNA-Phe*), and a very short portion of the 12S rRNA gene. Protocols for PCR amplification with the primers L14973 and H690 (Kumazawa et al., 1996) followed Újvári et al. (2005). These primers, additional published primers D543 and VuR1430 (Újvári et al., 2005), and two newly designed primers VuCRs1 (5′-TAATGTCCTTTCCAAGG-3′) and VuCRs2 (5′-TATCATCACCCTTCACA-3′) were used for sequencing. The new sequences were deposited in the GenBank database (Accession Numbers JN204695–JN204721).

2.2. Data analyses

The sequences were aligned by ClustalW (Thompson et al., 1994), as implemented in BioEdit 7.0 (Hall, 1999), and then checked by eye, resulting in an alignment of 2247 bp. Prior to analysis, several regions that were difficult to align were removed: positions 890–891 (adenosine residues in the *Cytb* stop codon in the sequences from Újvári et al. (2005)); positions 948 and 1698 (single-nucleotide insertions in *V. berus*), positions 980–1192 (STR-containing segment partly missing from the sequences from Újvári et al. (2005)). This resulted in a total of 2030 bp available for analysis.

Nucleotide substitution models for the use in the maximum likelihood (ML) phylogenetic analysis and in the analysis with the Bayesian approach (BA) were selected by the Akaike information criterion as implemented in jModelTest 0.1.1 (Posada, 2008) and in MrModeltest 2.3 (Nylander, 2004), respectively. The ML analysis was performed with PhyML 3.0 (Guindon et al., 2010) by using the best approach, which combines nearest neighbour interchanges with the subtree pruning and regrafting algorithm, and using the GTR + I + G model. Bootstrap values calculated from 1000 resampled datasets and the approximate likelihood-ratio test (aLRT; Anisimova and Gascuel, 2006) were used to assess the branch support. Bayesian analysis was performed with MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The analysis was set with partitions for genes (*Cytb*, *tRNA-Thr*, *CR1*, *tRNA-Phe*; the last one included the small portion of *12S rRNA*) and also for codon positions in *Cytb*. The likelihood settings corresponded to the best-fit model for each partition (*Cytb* pos1/pos2/pos3, HKY + I/HKY + I/GTR; *tRNA-Thr*, SYM + I; *CR1*, GTR + I + G; *tRNA-Phe*, HKY), with parameters optimized during the run. Two independent BA analyses were performed to check convergence, each with four coupled chains that were run for six

Table 1
Specimens examined.

Map	Taxon	Individual/GenBank	Country	Locality	Source
1	*V. ursinii ursinii*	AY311383	France	Vaucluse	Garrigues et al. (2005)
2		FM955066	France	Alpes Maritimes	Ferchaud et al. (2011)
3		FM955139	Italy	Gran Sasso d'Italia	Ferchaud et al. (2011)
4	*V. ursinii macrops*	V29/JN204695	Croatia	Velebit Mts.	This study
5	*V. ursinii rakosiensis*	AY300756	Hungary	Bugac, Kiskunság	Újvári et al. (2005)
		AY300757	Hungary	Bugac, Kiskunság	Újvári et al. (2005)
		AY300758	Hungary	Bugac, Kiskunság	Újvári et al. (2005)
6		V1/JN204696	Romania	Alba, Transylvania	This study
		V2/JN204697	Romania	Alba, Transylvania	This study
		V3/JN204698	Romania	Alba, Transylvania	This study
7	*V. ursinii moldavica*	V4/JN204699	Romania	Iaşi, Moldavia	This study
		V5/JN204700	Romania	Iaşi, Moldavia	This study
8		V6/JN204701	Romania	Danube Delta	This study
		V7/JN204702	Romania	Danube Delta	This study
9		V34/JN204703	Rep. of Moldova – pet trade	Tiraspol	This study
10	*V. renardi renardi*	V17/JN204704	Rep. of Moldova – pet trade	Tiraspol	This study
		V18/JN204705	Rep. of Moldova – pet trade	Tiraspol	This study
11		V21/JN204706	Ukraine	Kinburnska Kosa	This study
		V22/JN204707	Ukraine	Kinburnska Kosa	This study
		V23/JN204708	Ukraine	Kinburnska Kosa	This study
		V24/JN204709	Ukraine	Kinburnska Kosa	This study
12		AY300763	Ukraine	Yagorlitsky Kut	Újvári et al. (2005)
		AY300764	Ukraine	Yagorlitsky Kut	Újvári et al. (2005)
		AY300765	Ukraine	Yagorlitsky Kut	Újvári et al. (2005)
		AY300766	Ukraine	Yagorlitsky Kut	Újvári et al. (2005)
13		AY300759	Ukraine	Crimea	Újvári et al. (2005)
		AY300760	Ukraine	Crimea	Újvári et al. (2005)
		AY300761	Ukraine	Crimea	Újvári et al. (2005)
		AY300762	Ukraine	Crimea	Újvári et al. (2005)
14		V31/JN204710	Ukraine – pet trade	Kharkiv	This study
		V32/JN204711	Ukraine – pet trade	Kharkiv	This study
15		V9/JN204712	Russia – pet trade	Rostov na Donu	This study
		V10/JN204713	Russia – pet trade	Rostov na Donu	This study
		V19/JN204714	Russia – pet trade	Rostov na Donu	This study
16	*V. renardi eriwanensis*	V27/JN204715	Turkey	Arpaçay, Lesser Caucasus	This study
		V28/JN204716	Turkey	Arpaçay, Lesser Caucasus	This study
		FN870958	Turkey	Arpaçay, Lesser Caucasus	Ferchaud et al. (2011)
17	*V. renardi lotievi*	V12/JN204717	pet trade	Greater Caucasus	This study
		V14/JN204718	pet trade	Greater Caucasus	This study
18	*V. renardi tienshanica*	V20/JN204719	Kazakhstan – pet trade	Eastern Kazakhstan	This study
Outgroup	*V. kaznakovi*	V36/JN204720	Turkey – pet trade	Hopa	This study
Outgroup	*V. berus*	V8/JN204721	Czech Republic	Heřmanovice, Jeseníky Mts.	This study

million generations. Parameter and tree samples were saved every 100 generations and a 50% majority-rule consensus tree was constructed from the sampled trees after discarding the first 1/10 of trees as the burn-in.

Model-corrected and uncorrected *p*-distances among the sequences were calculated with PAUP* 4.0b10 (Swofford, 2003) and they were averaged between the taxa with MEGA 5.05 (Tamura et al., 2011), separately for *Cytb* (889 bp) and *CR1* (1006 bp) segments. The best-fit models for the two datasets were TIM1 + I + G for *Cytb* and TVM + I + G for *CR1*.

3. Results and discussion

The maximum-likelihood and Bayesian analyses yielded essentially the same tree topology (Fig. 2). The sequences from *V. ursinii* form a clade that is the sister clade to that of *V. renardi*. The clades are well-supported and the corrected distance between them is 6.1% for *Cytb* (4.7% *p*-distance) and 2.9% (2.5%) for *CR1*. This is in agreement with the opinion that *V. ursinii* and *V. renardi* are distinct species (Joger and Dely, 2005). If *V. renardi* was a subspecies of *V. ursinii* instead of a separate species (e.g. Újvári et al., 2005), we would expect *V. renardi* phylogenetically nested within *V. ursinii* and the genetic distance between them should be no greater than among subspecies (Table 2).

Within *V. ursinii* clade, the two montane subspecies, *V. u. ursinii* and *V. u. macrops*, form one clade and the lowland subspecies, *V. u. rakosiensis* and *V. u. moldavica*, another clade. The sequences from the recently discovered Romanian Alba population are grouped together with the sequences of *V. u. rakosiensis* from Kiskunság in Hungary, clearly separate from the sequences of *V. u. moldavica* from Iaşi in northeastern Romania, which are placed together with the sequences from the Danube Delta (Fig. 2). We found no haplotypes from the *V. renardi* clade among the vipers from Romania as would be expected if some of the populations had hybridized

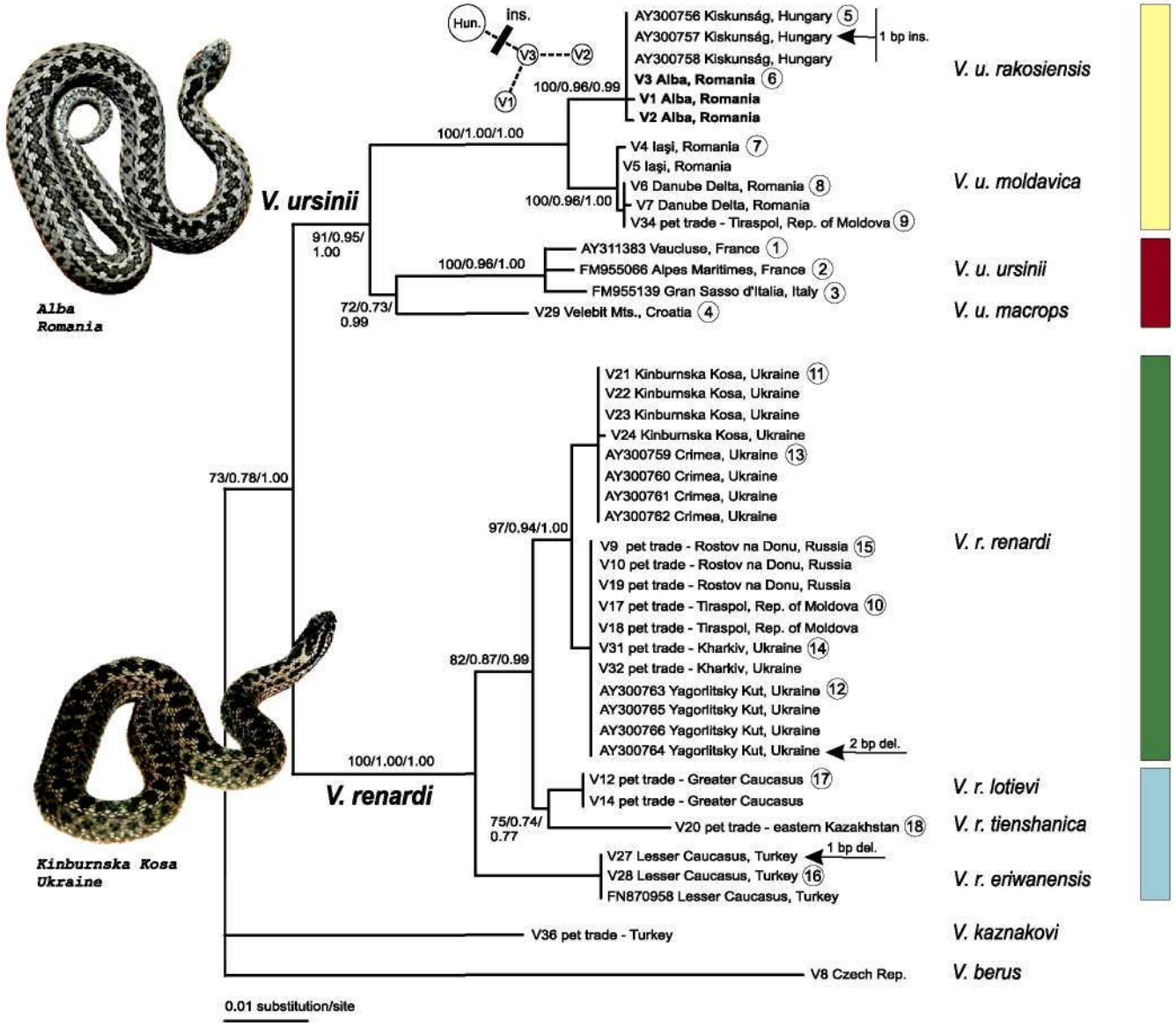

Fig. 2. Maximum-likelihood phylogeny of the meadow and steppe vipers. Statistical support for the major clades is expressed as the percentage bootstrap values, approximate likelihood ratio probabilities and Bayesian posterior probabilities. The inset (top) shows a hand-drawn haplotype network for *Vipera u. rakosiensis* from Alba (V1–V3) and Kiskunság (Hun.). The vertical bars (right) show habitat differences (see Fig. 1 for colour key). Numbers within the circles following localities (shown only once for each locality) correspond to sites in Fig. 1.

Table 2
Average uncorrected/model-corrected genetic distances among the species and subspecies from *Cytb* (below diagonal) and *CR1* data (above diagonal).

	V. ursinii rakosiensis	*V. ursinii moldavica*	*V. ursinii ursinii*	*V. ursinii macrops*	*V. renardi renardi*	*V. renardi lotievi*	*V. renardi tienshanica*	*V. renardi eriwanensis*	*V. kaznakovi*	*V. berus*
V. ursinii rakosiensis	–	0.7/0.7	–	1.6/1.8	2.3/2.6	2.4/2.8	3.2/3.9	2.3/2.7	3.1/3.7	4.7/7.0
V. ursinii moldavica	1.0/1.1	–	–	1.6/1.8	2.5/3.0	2.8/3.4	3.4/4.2	2.7/3.3	3.1/3.9	4.9/7.4
V. ursinii ursinii	3.8/4.7	3.8/4.7	–	–	–	–	–	–	–	–
V. ursinii macrops	4.2/5.1	4.0/4.9	2.6/3.0	–	2.6/3.0	2.7/3.2	3.1/3.8	2.3/2.6	2.7/3.2	4.6/6.7
V. renardi renardi	4.9/6.4	4.9/6.3	4.2/5.2	4.1/5.1	–	0.8/0.8	1.8/2.0	1.7/1.9	2.9/3.4	5.7/8.7
V. renardi lotievi	4.8/6.3	4.7/6.1	4.3/5.2	4.2/5.1	0.7/0.7	–	1.4/1.6	1.7/1.9	3.1/3.8	5.7/8.9
V. renardi tienshanica	5.1/6.6	4.9/6.4	4.4/5.5	4.4/5.4	1.1/1.1	0.9/0.9	–	2.0/2.3	3.4/4.3	6.1/10.0
V. renardi eriwanensis	5.2/6.8	5.3/6.9	4.9/6.2	4.9/6.3	1.7/1.8	1.6/1.7	2.0/2.2	–	3.0/3.7	5.3/8.2
V. kaznakovi	5.4/7.3	5.9/8.1	4.9/6.5	4.6/5.8	4.7/6.1	5.3/6.9	5.3/6.9	5.6/7.4	–	5.4/8.2
V. berus	5.8/7.8	5.8/7.7	5.3/6.9	5.5/7.1	5.0/6.3	5.3/6.7	5.7/7.4	5.6/7.2	4.4/5.3	–

(Nilson and Andrén, 2001; Zamfirescu et al., 2009). This supports the recognition of *V. u. moldavica* as a subspecies of *V. ursinii*, and it shows that the vipers from the Danube Delta also belong to *V. u. moldavica*, as suggested by Nilson and Andrén (2001) and Dely and Joger (2005) based on morphological similarity.

One of the vipers (V3) from Alba carried a haplotype that was identical with the published *CR1* sequence of *V. u. rakosiensis* from Kiskunság except that it lacked a 1-bp insertion unique to the Hungarian vipers (Fig. 2), and the other two Alba vipers had a very similar sequence to the first one (each different by only one nucleotide from it). Our analyses of mtDNA thus demonstrate that the vipers from the population discovered in Romania in 2002 (Ghira, 2007; Krecsák and Zamfirescu, 2008) most likely are *V. u. rakosiensis*. Although our results need corroboration from additional molecular markers (e.g. Ferchaud et al., 2011), we did not find any evidence that would suggest that the Alba population hybridized with either *V. u. moldavica* or *V. renardi*. It therefore appears to be the third last surviving population of *V. u. rakosiensis* and the only known population of this subspecies outside Hungary. It is remarkable that the Alba and Kiskunság populations share a very similar mtDNA sequence, despite the geographical distance separating them (Fig. 1). Apparently, the populations were interconnected by gene flow until relatively recently, and lineage sorting has not yet led to reciprocal monophyly of their mtDNA, despite the small current census size of both populations (Cheylan et al., 2011; Edgar and Bird, 2006). *V. renardi* contains three distinct clades (Fig. 2), one of which consists of specimens collected from four sites spanning the range of *Vipera renardi renardi* in the lowlands of Ukraine and from a site in the southern Russia, and it also includes two of the three vipers from the pet trade collected near Tiraspol in the Republic of Moldova. This site is situated in the lowland steppe between Romania and Ukraine (Fig. 1), and it is interesting that the third viper from Tiraspol (V34) had the sequence of *V. u. moldavica* (Fig. 2), which suggests a hybrid origin or sympatric population (see Krecsák et al., 2003; Nilson and Andrén, 2001; Nilson et al., 1993), or that it might be a mislabeled specimen in the pet trade. The two montane subspecies of *V. renardi* from the Greater Caucasus and Tien-Shan, *V. r. lotievi* and *V. r. tienshanica*, form a clade, although not with high support, that is the sister clade to *V. r. renardi*. Finally, the montane subspecies from the Lesser Caucasus, *V. r. eriwanensis*, occupies a basal position in the *V. renardi* clade and forms the sister group to the rest of *V. renardi* (Fig. 2). Our data therefore would not appear to justify the distinction of *V. r. lotievi* as a separate species from *V. renardi* (e.g. David and Vogel, 2010; Nilson et al., 1995). Although it does form a distinct phylogenetic lineage, it is separated by much less genetic distance from the other subspecies of *V. renardi* than *V. renardi* is from *V. ursinii* (Fig. 2; Table 2). The same may be said of *V. r. eriwanensis*, which has often been considered a full species as well (e.g. David and Vogel, 2010; Ferchaud et al., 2011; Nilson and Andrén, 2001).

The fact that the montane subspecies of *V. ursinii* and *V. renardi* were placed in the 'correct' species clade with the conspecific lowland subspecies suggests that the ecological and altitudinal divergence have occurred at least once in each species (Fig. 2). The two lowland subspecies of *V. ursinii* form a well-supported clade, sister to the clade of the two montane subspecies, which refutes the close relationship of each lowland subspecies with a different montane subspecies (*V. u. rakosiensis* with *V. u. ursinii* and *V. u. moldavica* with *V. u. macrops*), suggested based on the immunological distance by Nilson et al. (1993) and based on morphology by Nilson and Andrén (2001). Instead, our mtDNA phylogeny is consistent with a single origin of the montane subspecies from a lowland ancestor (or vice versa!) in *V. ursinii*. In contrast, the three montane subspecies of *V. renardi* do not form a clade as *V. r. eriwanensis* is the sister group to the rest of *V. renardi* (Fig. 2). Thus, in this species, the colonization of the montane habitats by the widespread lowland ancestor most likely occurred twice in the Caucasus and independently in Central Asia (Fig. 1). The fact that *V. r. lotievi* and *V. r. tienshanica* are sister clades in our phylogeny suggests they are derived from lowland *V. renardi* that our sampling does not include (e.g. 'east-*renardi*' of Nilson and Andrén, 2001). In all cases, the montane clades are genetically too distinctive from the lowland clades (Table 2) to have evolved since the last glaciation (Avise et al., 1998), and their isolated occurrences in mountain ranges of the south of Europe and western and central Asia may therefore represent 'preglacial relics' related to local steppe habitats. There is, however, much less genetic distance between *V. u. ursinii* from the French Alps and from the Apennines, suggesting relatively recent divergence between the populations of this subspecies in the two mountain ranges. Our mitochondrial DNA phylogeny thus provides important new insights into the evolutionary history and systematics of meadow and steppe vipers, to be assessed with independent information from nuclear DNA, morphology and ecology.

The discovery of *V. u. rakosiensis* in Romania may increase the possibility for the conservation of the Hungarian populations. Given the close mtDNA similarity between the Kiskunság and Alba vipers, it may seem reasonable to suggest translocations of vipers from Alba to Hungary to restore the reduced genetic variation of the Hungarian populations (Újvári et al., 2002). Noteworthy in this context is that each of the three vipers from Alba carried a different haplotype (Fig. 2). However, the absence of mtDNA divergence between the two populations should not be taken to mean the absence of adaptive differences they may have acquired at other genes in response to local selective forces and which the translocations could disrupt. Therefore, any translocation programs should ideally be preceded by careful evaluation of the adaptive differentiation between the Hungarian and Romanian vipers.

Given their phylogenetic divergence and geographical and ecological isolation, the montane populations of both *V. ursinii* and *V. renardi* also should be considered of high conservation concern. The same applies to *V. u. moldavica*, which has a very small range

and is threatened by disappearing habitat (Edgar and Bird, 2006; Nilson et al., 1993; Zamfirescu et al., 2009).

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