Systematics and Biodiversity (2016)

Taylor & Francis
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Research Article

Cryptic diversity and unexpected evolutionary patterns in the meadow lizard, *Darevskia praticola* (Eversmann, 1834)

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(Received 4 December 2014; accepted 15 October 2015)

Darevskia praticola differs from the other species of the genus in having a large but disjunct distribution, covering the Balkan and the Caucasus regions. Furthermore, most *Darevskia* species occupy saxicolous habitats, whereas *D. praticola* inhabits meadows and forest environments. Here we determine the phylogeographic and phylogenetic relationships of *Darevskia praticola* sensu lato and evaluate the current, morphology-based taxonomy. We sequenced two mtDNA genes (Cyt-*b* and ND4) and two nuclear loci (MC1R and RELN) for samples collected across the species range. Because our sequences amplified with the Cyt-*b* primers appear to represent a nuclear pseudogene we excluded this marker from the final analysis. Our results support monophyly of *D. praticola* and show its division into three clades. The first divergence, dated to the Late Pliocene, is between the Balkans and the Caucasus. The Caucasus lineage is further subdivided in a western Greater Caucasus and a Transcaucasia clade, likely due to subsequent differentiation during the Pleistocene. Our findings do not support the current taxonomic arrangement within *D. praticola*. The main geographic divergence likely happened due to a vicariance event associated with Plio-Pleistocene climatic and vegetation oscillations.

Key words: Balkans, Caucasus, historical biogeography, phylogeny, phylogeography, mtDNA, nDNA, pseudo-gene

Introduction

Due to its turbulent geological history, the Black Sea region, encompassing the Caucasus and the Balkans, is characterized by a complex landscape and a great diversity of habitats. Plate tectonic movements, abrupt palaeoclimatic changes in the Plio-Pleistocene, changes in sea level and sea extension as well as the interconnections between the water basins surrounding the Black Sea and the Mediterranean created barriers to dispersal routes, allowing for the vicariance of species and biotas (Popov et al., 2006).

Phylogeographic studies of the herpetofauna in this region are scarce compared with those available for other West Palaearctic glacial refugia. Nevertheless, the studies performed so far reveal considerable complexity of vertebrate phylogeographic patterns (e.g. Fritz et al., 2009; García-Porta et al., 2009; Gvoždík et al., 2010; Lymberakis et al., 2007; Marosi et al., 2012; Mashkaryan et al., 2013; Recuero et al., 2011; Stöck et al., 2006, 2012;

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ISSN 1477-2000 print / 1478-0933 online

http://dx.doi.org/10.1080/14772000.2015.1111267.

Tarkhnishvilli, Murtskhvaladze, & Gavashelishvili, 2013; Ursenbacher et al., 2008; Wielstra et al., 2010), and suggest a dual role for the Black Sea region, where vertebrate species show different patterns of distribution. While some species show a disjunct distribution between the Balkans and the Caucasus, with their distribution range divided into different clades, others show a uniform distribution across the Black Sea region, linking the Balkans and the Caucasus (Fritz et al., 2009; Garcia-Porta et al., 2012; Gvoždík et al., 2010).

Darevskia is a genus of small lizards, occurring in a wide variety of environments from rocks to meadows and comprising 27 species (Arnold, Arribas and Carranza, 2007; Tarkhnishvilli, 2012). The most widespread species, *Darevskia praticola* (Eversmann, 1834), has a disjunct distribution and is found across the Caucasus, from the Black Sea coast to northwestern Iran, and in the eastern Balkans where it is the sole representative of the genus (Fig. 1). Unlike most *Darevskia* species, *D. praticola* is mainly a ground dweller, restricted to clearings with lush vegetation, meadows and glades within open broad-leaf woodland (Agasyan et al., 2009). Roitberg (1999) suggested that *D. praticola* has undergone a recent niche shift to forest habitat from rocky and stony habitats that appear to be ancestral for the genus.

Previously, the relationships between *D. praticola* and the other *Darevskia* species were inferred from a single mitochondrial DNA marker (Cyt-*b*) by Murphy, Darevsky, MacCulloch, Fu, and Kupriyanova (1996), and later by Tarkhnishvilli (2012) and Ahmadzadeh et al. (2013b). These works have placed *D. praticola* within the *saxicola* species group, as the sister taxon to *D. saxicola* and *D. brauneri*.

The taxonomy within *D. praticola* sensu lato is complex and remains controversial despite several taxonomic revisions (Tuniyev, Doronin, Kidov & Tuniyev, 2011, 2013 and references therein), which were mainly based on scalation and mostly focused on Caucasian populations. The most recent studies (Ilgaz & Kumlutaş, 2005; Ljubisavljevic et al., 2006, 2008; Tuniyev et al., 2011) identified four taxa with allopatric distributions. The nominate subspecies (*D. p. praticola*) is found in the northeastern Caucasus and Transcaucasia. *Darevskia praticola pontica* (Lantz & Cyrén, 1919) occupies the northwestern Caucasus and the Balkans (southeastern Romania, Serbia, Greece, and Bulgaria) and Turkish Thrace (jointly referred as Balkans below). *Darevskia praticola hungarica* (Sobolevsky 1930) originates from the Transylvanian Alps in Romania and is currently synonymised with *D. praticola pontica*. Tuniyev et al. (2011) assigned a lectotype and paralectotypes from the northwestern Caucasus for *D. pontica* and described the subspecies *D. praticola hyrcanica*. *Darevskia praticola hyrcanica* (Tuniyev et al., 2011) is restricted to the southwest of the Caspian Sea region: the Talysh mountains, Anzali Bay, and Western Elburz mountains (Tuniyev et al., 2011). Finally, *D. praticola loriensis* (Tuniyev, Doronin, Tuniyev, Aghasyan, Kidov & Aghasyan, 2013) was recently described from Armenia (Tuniyev et al., 2013).

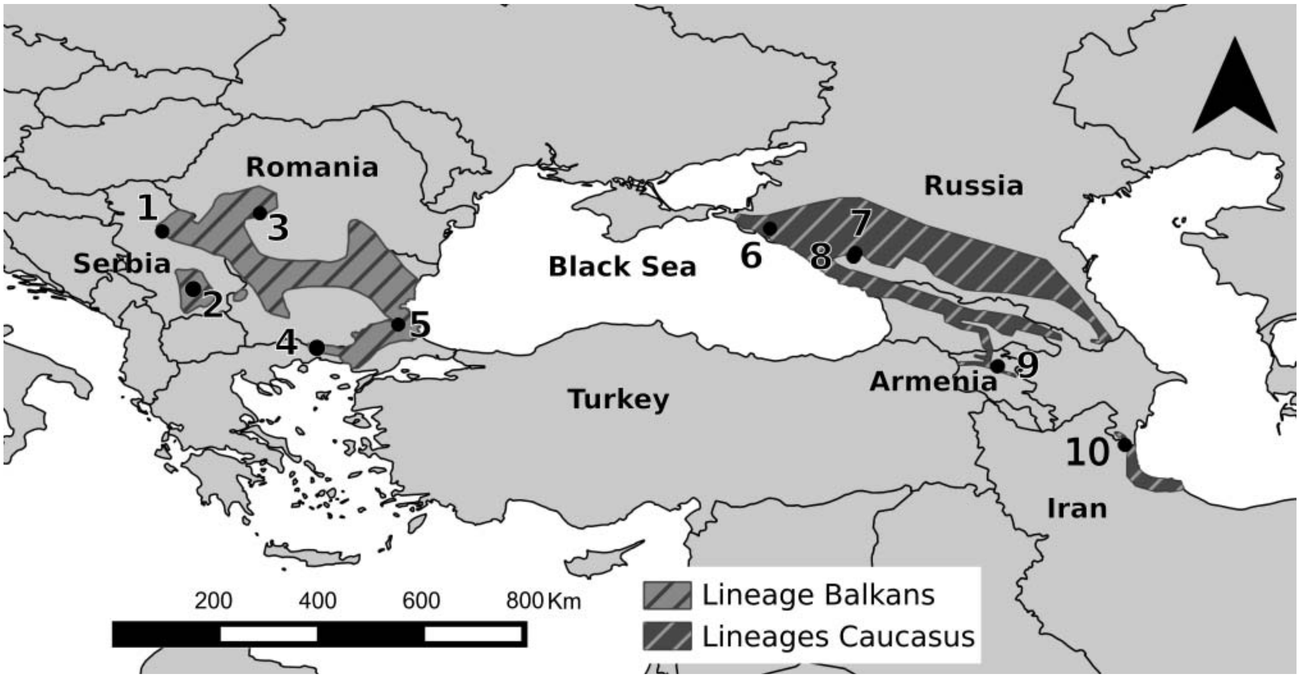

Fig. 1. Distribution of *Darevskia praticola* sensu lato. The Balkan lineage is shown in light grey and the two Caucasus lineages in dark grey. Population numbers correspond to Table 1. Distribution is based on Valakos et al. (2008) and Agasyan et al. (2009).

The Balkan population is currently synonymized with *D. p. praticola* from the Caucasus because of a few shared scalation characters (Tuniyev et al., 2011, 2013). However, due to their disjunct distribution and frequent occurrence of homoplasy in lacertid scalation (e.g. Doan & Castoe, 2005; Johnston et al., 2013; Wiens, Brandley, & Reeder, 2006), some authors argue that the Balkan populations should be recognized as a separate species (Stugren 1961; Bischoff 1976). Unfortunately, a range-wide genetic study that could elucidate the relationships between *D. praticola* subspecies and disjunct populations is still lacking (Ljubisavljevic et al., 2006; Tuniyev et al., 2011).

Here, we use multilocus sequence data and range-wide sampling to determine the interspecific and intraspecific phylogenetic relationships of *D. praticola* and discuss taxonomic implications of our findings. Molecular dating is applied to allow inference of historical biogeographic scenarios for *D. praticola*.

Materials and methods

We sampled 31 *D. praticola* specimens, which were collected between 1996 and 2012, from across the distribution range (Table 1, Fig. 1). Tail tips were preserved in absolute ethanol and photos were taken for most of the individuals collected, after which lizards were released at their collection sites. Animals were not killed and no voucher specimens were collected.

Table 1. Sampled individuals and haplotypes for the MC1R and RELN nuclear markers. Taxonomy according to Tuniyev et al. (2011, 2013) and Ljubisavljevic et al. (2006).

Code	Species	Subspecies	Country	Lat	Lon	MC1R	ReLN	Map
Balkans								
5201	*D. praticola*	*pontica*	Greece	41.33	24.88	5,5	-,-	4
5175	*D. praticola*	*pontica*	Romania	45.2180	23.3938	5,5	7,7	3
5176	*D. praticola*	*pontica*	Romania	45.2180	23.3938	5,5	7,7	3
5183	*D. praticola*	*pontica*	Romania	45.2180	23.3938	5,5	7,7	3
5204	*D. praticola*	*pontica*	Romania	45.2180	23.3938	-,-	7,7	3
13283	*D. praticola*	*pontica*	Serbia	44.6829	20.5528	3,4	5,5	1
13285	*D. praticola*	*pontica*	Serbia	44.6829	20.5528	3,3	-,-	1
13293	*D. praticola*	*pontica*	Serbia	44.6829	20.5528	4,4	5,6	1
13297	*D. praticola*	*pontica*	Serbia	44.6824	20.5528	3,3	5,6	1
13294	*D. praticola*	*pontica*	Serbia	44.6815	20.5518	3,3	6,6	1
13297	*D. praticola*	*pontica*	Serbia	44.6824	20.5528	3,3	5,6	1
5200	*D. praticola*	*pontica*	Serbia	44.6822	20.5521	3,3	5,6	1
5177	*D. praticola*	*pontica*	Serbia	43.2895	21.3070	-,-	5,7	2
16331	*D. praticola*	*pontica*	Turkey	41.9718	27.4232	5,5	7,7	5
Caucasus								
10360	*D. praticola*	*loriensis*	Armenia	40.7623	44.8885	1,1	1,1	9
6279	*D. praticola*	*loriensis*	Armenia	40.7500	44.8667	1,1	8,8	9
13916	*D. praticola*	*hyrcanica*	Azerbaijan	38.4667	48.5833	1,1	-,-	10
13917	*D. praticola*	*hyrcanica*	Azerbaijan	38.4667	48.5833	1,1	2,2	10
13918	*D. praticola*	*hyrcanica*	Azerbaijan	38.4667	48.5833	1,1	2,2	10
12191	*D. praticola*	*pontica*	Russia	44.0574	40.7342	1,2	3,3	7
12192	*D. praticola*	*pontica*	Russia	44.0574	40.7342	2,2	-,-	7
12193	*D. praticola*	*pontica*	Russia	44.0574	40.7342	1,1	-,-	7
12194	*D. praticola*	*pontica*	Russia	44.0574	40.7342	1,2	4,4	7
12195	*D. praticola*	*pontica*	Russia	44.0574	40.7342	1,1	3,3	7
12196	*D. praticola*	*pontica*	Russia	44.0574	40.7342	2,2	3,3	7
12197	*D. praticola*	*pontica*	Russia	44.0574	40.7342	1,1	3,3	7
12198	*D. praticola*	*pontica*	Russia	44.0574	40.7342	1,2	-,-	7
14664	*D. praticola*	*pontica*	Russia	43.9500	40.6833	1,1	3,3	8
14665	*D. praticola*	*pontica*	Russia	43.9500	40.6833	1,2	3,3	8
14666	*D. praticola*	*pontica*	Russia	43.9500	40.6833	1,2	3,3	8
6300	*D. praticola*	*pontica*	Russia	44.7667	38.2500	1,1	9,9	6

Total genomic DNA was extracted from approximately 30 mg of tissue following standard high-salt protocols (Sambrook and Green, 2000). DNA was eluted with Elution buffer (Qiagen). For older samples the DNeasy Blood and Tissue kit (Qiagen) was used.

Two mtDNA genes Cytochrome-*b* (Cyt-*b*) and the 3′ end of the NADH dehydrogenase subunit 4 gene and adjacent tRNAs (ND4) and two nDNA genes (melanocortin-1 receptor: MC1R; Intron 61 of the reelin gene: RELN) were sequenced. Primers were described in Kocher et al. (1989), Arevalo et al. (1994) and Pinho, Harris and Ferrand (2009). Amplifications were performed in 25 μL of 5× reaction buffer, 3.2 mM MgCl2, 1.6 mM each dNTP, 4.0 M each primer, 1U of Promega GoTaq DNA polymerase. PCRs consisted of an initial denaturation at 94°C for 2 min, followed by 40 cycles of denaturation step at 92°C for 30″, annealing at 57−65°C for 30″ and extension at 72°C for 1’. A final extension was conducted at 72°C for 5 min.

Sequencing was performed commercially (Macrogen® Europe). Sequences were edited with Chromas Pro v1.7.6 (Technelysium Pty, Ltd) and aligned with Mafft version 7 (Katoh & Standley, 2013), using default options for all the alignments except for RELN, for which the iterative refinement method E-INS-i was used. This algorithm is better suited for sequences with multiple conserved domains and long indels, as we expect for the RELN dataset. Heterozygous MC1R sequences were separated using PHASE 2.1.1 (Stephens, Smith, & Donnelly, 2001; Stephens & Scheet 2005) with SeqPhase (Flot 2010). The probability for every haplotype chosen was between 0.978 and 1.00. All new sequences were submitted to GenBank (accession numbers pending), and the alignments are available from Dryad (reference number pending). Nucleotide diversity parameters were calculated with DNAsp (Librado & Rozas, 2009). Genetic uncorrected *p*-distances were calculated using MEGA6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013).

We performed a first phylogenetic analysis for each of the mtDNA markers (Cyt-*b* and ND4) with an extended dataset. Sequences from other *Darevskia* sp. taken from our database, Ahmadzadeh et al. (2013) and available from GenBank (Table 2), were included (chosen according to phylogenetic proximity to ingroup and availability) (Appendix 1, GenBank codes and accession numbers, see online supplemental material, which is available from the article’s Taylor & Francis Online page at http://dx.doi.org/10.1080/14772000.2015.1111267). The best-fit evolutionary model for each gene (Cyt-*b*, 1st codon: GTR+IG; Cyt-*b*, 2nd codon: GTR+G; Cyt-*b*, 3rd codon: HKY+IG; ND4, 1st codon: GTR+G; ND4, 2nd codon: HKY+G; ND4, 3rd codon: GTR+IG; ND4, tRNA: GTR+G; MC1R: TPM3uf+G) were selected in PartitionFinder (Cyt-*b* and ND4) (Lanfear et al., 2012) and

Table 2. Samples analysed for MC1R and Cyt-*b* analyses for other *Darevskia* species. Taxonomy according to Tuniyev et al. (2011, 2013) and Ljubisavljevic et al. (2006).

MC1R haplotype	DB Code	Species	Subspecies[1]	Country
1	10360	*D. praticola*	*loriensis*	Armenia
1	13916	*D. praticola*	*hyrcanica*	Azerbaijan
1	13917	*D. praticola*	*hyrcanica*	Azerbaijan
1	13918	*D. praticola*	*hyrcanica*	Azerbaijan
1	14664	*D. praticola*	*pontica*	Russia
1	14665	*D. praticola*	*pontica*	Russia
1	14666	*D. praticola*	*pontica*	Russia
1	6279	*D. praticola*	*loriensis*	Armenia
1	6300	*D. praticola*	*pontica*	Russia
1	12191	*D. praticola*	*pontica*	Russia
1	12193	*D. praticola*	*pontica*	Russia
1	12194	*D. praticola*	*pontica*	Russia
1	12195	*D. praticola*	*pontica*	Russia
1	12197	*D. praticola*	*pontica*	Russia
1	12198	*D. praticola*	*pontica*	Russia
2	13283	*D. praticola*	unknown	Serbia
2	13285	*D. praticola*	unknown	Serbia
2	13294	*D. praticola*	unknown	Serbia
2	13297	*D. praticola*	unknown	Serbia
2	5200	*D. praticola*	unknown	Serbia
3	13283	*D. praticola*	unknown	Serbia
3	13293	*D. praticola*	unknown	Serbia
4	14665	*D. praticola*	*pontica*	Russia
4	14666	*D. praticola*	*pontica*	Russia
4	12191	*D. praticola*	unknown	Russia
4	12192	*D. praticola*	unknown	Russia
4	12194	*D. praticola*	unknown	Russia
4	12196	*D. praticola*	unknown	Russia
4	12198	*D. praticola*	unknown	Russia
5	16331	*D. praticola*	unknown	Turkey
5	5175	*D. praticola*	unknown	Romania
5	5176	*D. praticola*	unknown	Romania
5	5183	*D. praticola*	unknown	Romania
5	5201	*D. praticola*	unknown	Greece
6	5392	*D. rudis*		Turkey
6	5402	*D. rudis*		Turkey
6	5403	*D. rudis*		Turkey
6	5250	*D. rudis*		Turkey
7	5402	*D. rudis*		Turkey
7	5403	*D. rudis*		Turkey
8	7802	*D. derjugini*		Georgia
9	7803	*D. derjugini*		Georgia
9	7846	*D. derjugini*		Georgia
10	7803	*D. derjugini*		Georgia
11	13797	*D. kopetdaghica*		Iran
11	13798	*D. kopetdaghica*		Iran
12	13807	*D. schaekeli*		Iran
13	13818	*D. kamii*		Iran

(*continued*)

Table 2. (*Continued*)

MC1R haplotype	DB Code	Species	Subspecies[1]	Country
13	13819	*D. kamii*		Iran
13	6150	*D. caspica*		Iran
13	6151	*D. caspica*		Iran
14	13818	*D. kamii*		Iran
15	6146	*D. kamii*		Iran
16	6111	*D. steineri*		Iran
16	6112	*D. steineri*		Iran
16	6115	*D. steineri*		Iran
17	6117	*D. chlorogaster*		Iran
17	6118	*D. chlorogaster*		Iran
18	6200	*D. defilippii*		Iran
18	6201	*D. defilippii*		Iran
18	13799	*D. defilippii*		Iran
19	6316	*D. defilippii*		Iran
20	10197	*D. raddei*		Armenia
21	10197	*D. raddei*		Armenia
22	10019	*D. raddei*		Armenia
22	*LSH1*	*D. raddei*	*nairensis*	Armenia
23	*LSH1*	*D. raddei*	*nairensis*	Armenia
24	LSH3	*D. valentini*		Armenia
24	LSH4	*D. valentini*		Armenia
25	LSH3	*D. valentini*		Armenia
25	LSH4	*D. valentini*		Armenia
26	ST2	*D. portschinskii*	*portschinskii*	Armenia
26	10071	*D. portschinskii*	*portschinskii*	Armenia

JMODELTEST v0.1.1 (MC1R) (Posada, 2008) using the corrected Akaike information criterion.

Bayesian phylogenetic analysis for this dataset and for each marker (Cyt-*b* and ND4) was conducted with MrBayes v3.2 (Ronquist et al., 2011), using four heated 20 million generation Markov chains (default heating values) sampled every 1000 generations. Each run was performed twice, to allow identification of suboptimal runs. These two sets were later combined. The first 6000 trees were discarded as burn-in after analysing the convergence parameters for both runs in Tracer v1.4 (Rambaut & Drummond, 2007). Maximum likelihood (ML) analyses were performed using MEGA6 (Tamura et al, 2013) and branch support was estimated using the bootstrap technique with 500 replicates (Felsenstein, 1985).

A multispecies coalescent model (Heled & Drummond 2010) implemented in *BEAST v1.8 was used to infer the species tree from multiple gene trees for the major lineages in *D. praticola* identified from mtDNA. In the multispecies coalescent model all model parameters are unlinked across loci, including the topology parameter, which allows the gene trees to differ in topology, even though being constrained by the species tree. Therefore, this approach takes into account gene tree heterogeneity due to incomplete lineage sorting and different evolutionary rates. The dataset consisted of all the *D. praticola* individuals used in previous analyses. We used five *Darevskia* individuals as outgroups: one *D. portschinskii* (TZ1), one *D. raddei* (10126) and three *D. derjugini* (7802, 7803, 7846).

Since there was some evidence suggesting that Cyt-*b* could be a mix of mtDNA and nuclear pseudogene sequences (see Results) this gene was discarded from the analyses and only three loci ND4 (mtDNA), MC1R (nuclear) and RELN (nuclear) were included.

The time of the most recent common ancestor (tMRCA) was estimated in the species tree analysis. This was set to run for 10^8 generations, sampling every 10^5 generation, and repeated eight independent times. An uncorrelated lognormal relaxed clock was used for the mtDNA and nuclear markers. Individuals were assigned to different trait groups in *BEAST based on the previous results on the individual markers analysis. So, *D. praticola* individuals were partitioned according to the ND4 Bayesian and ML phylogeny and the MC1R and RELN network structures.

The prior for the ucld.mean parameter for the ND4 dataset was set as a normal distribution with a mean of 0.0226 and a standard deviation of 0.0031, so that mutation rate would be able to vary between 0.0278 and 0.0174 mutation/site/million years. This mutation rate interval was estimated for the ND4 gene in the lizard genus *Podarcis* (Pinho et al., 2007). LogCombiner (Drummond et al., 2012) was used to compile the log and tree files of the eight runs. 10% of the trees were discarded as burn-in, following an analysis of convergence of individual run parameters in Tracer v1.4 (Rambaut & Drummond, 2007). The best-fit evolutionary model for non-partitioned gene (ND4: TrN+G; MC1R: TPM3uf+G; RELN: HKY+I) was selected in JMODELTEST v0.1.1 (Posada 2008) using the corrected Akaike information criterion. BEAST v1.8 does not include all models present in JModeltest, thus evolutionary models applied were those available in BEAST which best approximated those selected by jModeltest (ND4: HKY+G; MC1R: GTR+GI; RELN: HKY+I). The Yule process of speciation was selected as a tree prior with a random starting tree. High effective sample sizes were observed for all parameters (posterior ESS values > 200 for the combined analyses). Convergence for all model parameters was determined in Tracer when sample size (ESS) > 200. Maximum clade credibility trees with divergence time means and 95% highest probability densities (HPDs) were produced using Tree Annotator (Drummond et al., 2012).

After phasing all haplotypes, a statistical parsimony network was built in TCS v1.21 (Clement et al., 2000) for the MC1R marker, with *D. praticola* and other *Darevskia* species for which data were available on GenBank. For the RELN gene, a median joining approach implemented

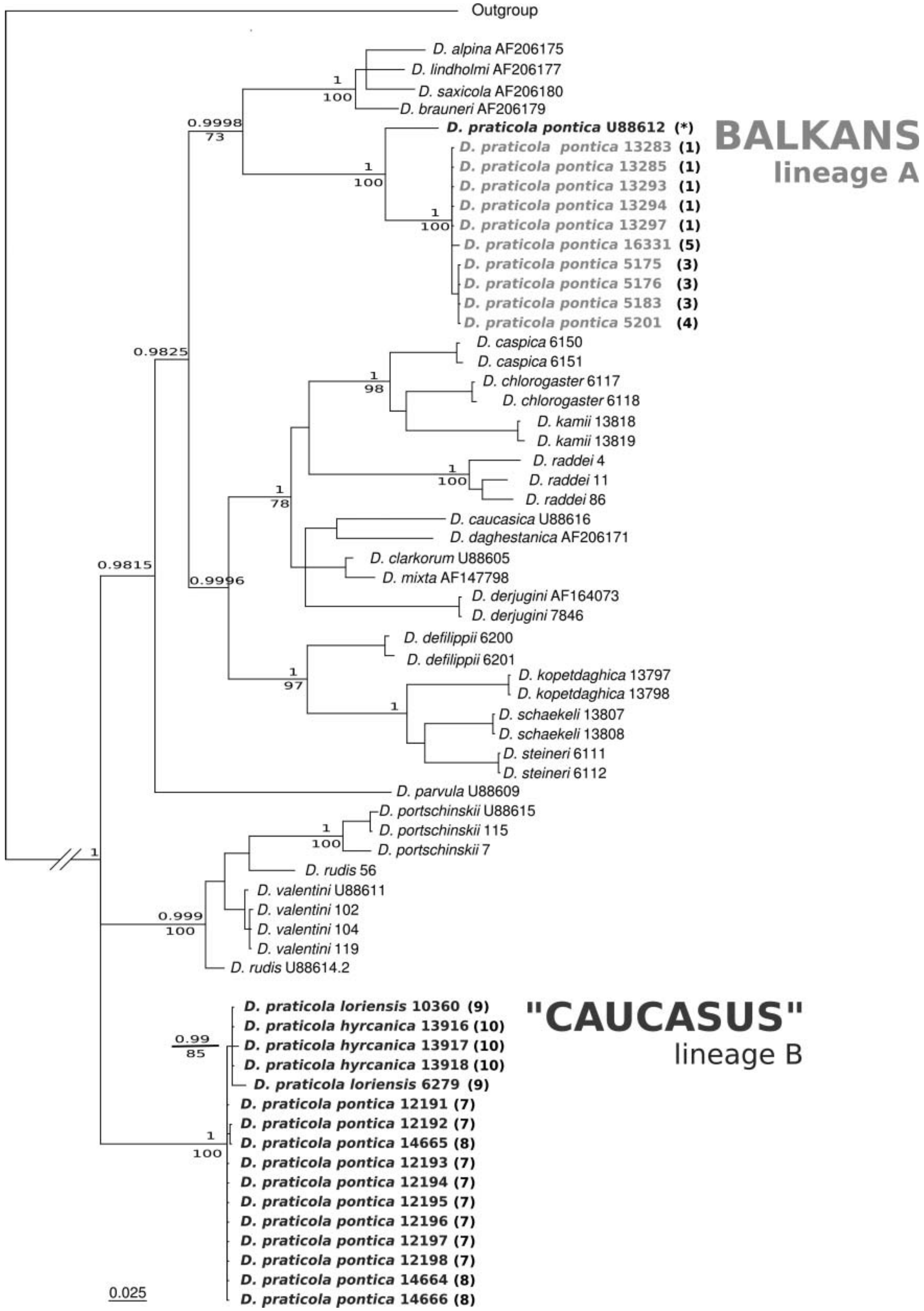

Fig. 2. Bayesian and ML phylogeny based on the mtDNA gene Cyt-*b* for an enlarged species dataset of the genus *Darevskia*. The Balkan lineage includes all the samples from the Balkans and is sister to the sample U88612 from the Caucasus. The other samples from the Caucasus belong to a single lineage of the basal trichotomy. The basal trichotomy in the consensus tree is only found in the Bayesian Cyt-*b* tree while in the ML tree the corresponding branch does not show a significant support (lower than 40/100). Numbers inside parentheses correspond to the locality number in Fig. 1.

in Network was used instead. This software was chosen given the high number of indels present in RELN and since it allows using indels as a 5th state (Bandelt et al., 1999).

Results

Phylogenetic analysis

Phylogenetic analyses produced distinctly different topologies for the mtDNA markers (Figs 2, 3). In the Cyt-*b* tree (Fig. 2), *D. praticola* is not monophyletic and is divided into two highly divergent clades. The phylogeny shows a basal trichotomy. One lineage includes a majority of *Darevskia* species, *D. praticola* from the Balkans, and a single individual from the Caucasus. The distance between the latter individual and the Balkan clade of *D. praticola* is 4.6% (*p*-distance = 0.046). A second lineage includes *D. portschinskii*, *D. rudis* and *D. valentini*. The third lineage includes the remaining *D. praticola* samples from the Caucasus and shows low intralineal variation. The divergence between the two *D. praticola* clades is over 12% (*p*-distance = 0.125). Base composition differed between Balkan and Caucasus individuals (data not shown). The third lineage was considered to be a pseudogene (see discussion) and excluded from further analysis.

There are three main clades within *D. praticola* ND4 tree (Fig. 3), which are less divergent than the Cyt-*b* clades. The Greater Caucasus and Transcaucasia (Armenia and Azerbaijan) clades are sisters forming a larger Caucasian clade which, in turn, is sister to the Balkan clade. The divergence between the Caucasian and the Balkan clades corresponds to an average 4.4% (*p*-distance = 0.044).

The Greater Caucasus clade included 12 individuals from the western Greater Caucasus (Krasnodarskiy Krai, Russian Federation). The Transcaucasian clade includes three individuals from the Talysh Mountains (Astara District, Azerbaijan, locality 10 in Fig. 1) and two individuals from the Lesser Caucasus (Tavush Marz, Armenia, locality 9 in Fig. 1). The Balkan clade includes seven individuals from two localities in Serbia (approximately 200−250 km apart, localities 1 and 2 in Fig. 1), three from one locality in Romania, one from Greece and one from Turkish Thrace (in Fig. 1, localities 3, 4 and 5, respectively).

The sister relationship of the two Caucasian clades and their monophyly with the Balkan clade were strongly supported (posterior probability of 1 and 99% bootstrap for the *D. praticola* Caucasus vs Balkans split and 1 posterior probability and 79% bootstrap for the Caucasus split).

The median joining analysis for the RELN gene recovers a network of nine haplotypes based on 37 polymorphic and 36 parsimony informative sites of 774 bp (Fig. 4). Three haplotypes (5−7) are found only in the Balkans and differ by 2−4 substitutions from each other. Haplotypes 1, 2, and 8 are found in Armenia and Iran and form another closely related group of haplotypes that differ by one or two mutations from each other. Although distant from each other in the network, haplotypes 3, 4, and 9 are found in the Western Greater Caucasus.

The MC1R statistical parsimony network (Fig. 5) identified five haplotypes in *D. praticola* based on six polymorphic and six parsimony informative sites of 620 bp. Haplotype 1 is found in the Western Greater Caucasus,

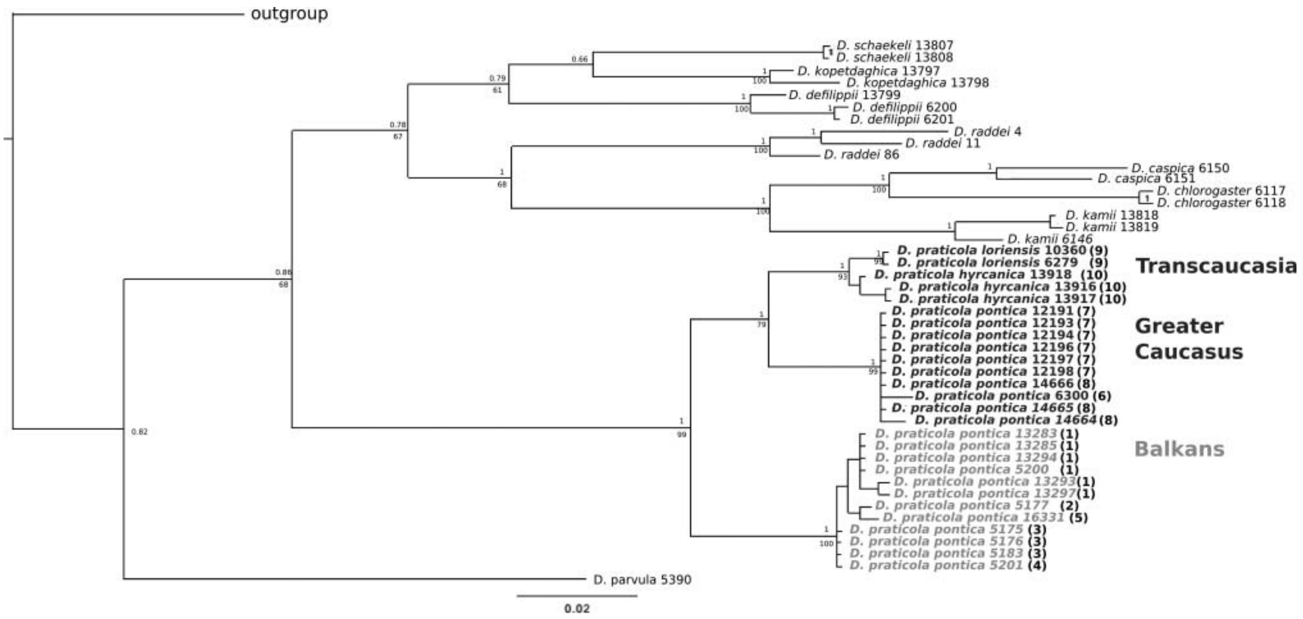

Fig. 3. Bayesian and ML phylogeny based on the mtDNA gene ND4 for *D. praticola* and outgroup species.

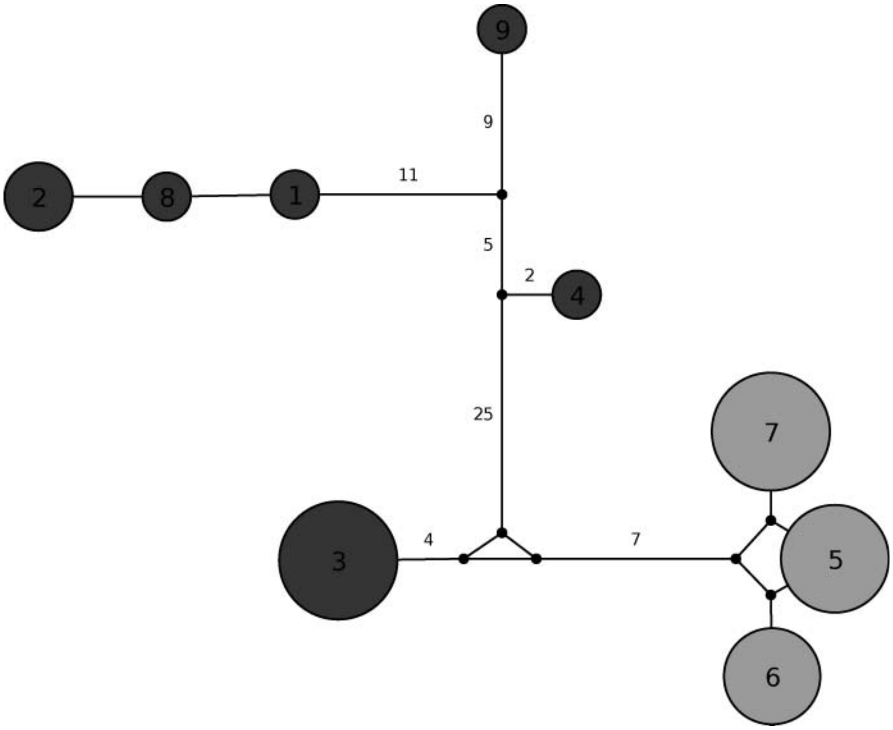

Fig. 4. Median-joining network for the nuclear gene RELN, based on the sequencing of 23 individuals of *D. praticola*. Number of substitutions is shown along the network branches. If branches are not noted, the corresponding haplotypes are separated by a single step. Small black circles correspond to the median vectors. Light grey represents the Balkan lineage and dark grey the Caucasian lineage. Circles correspond to haplotypes, numbered as in Table 1, with size reflecting the number of individuals per haplotype.

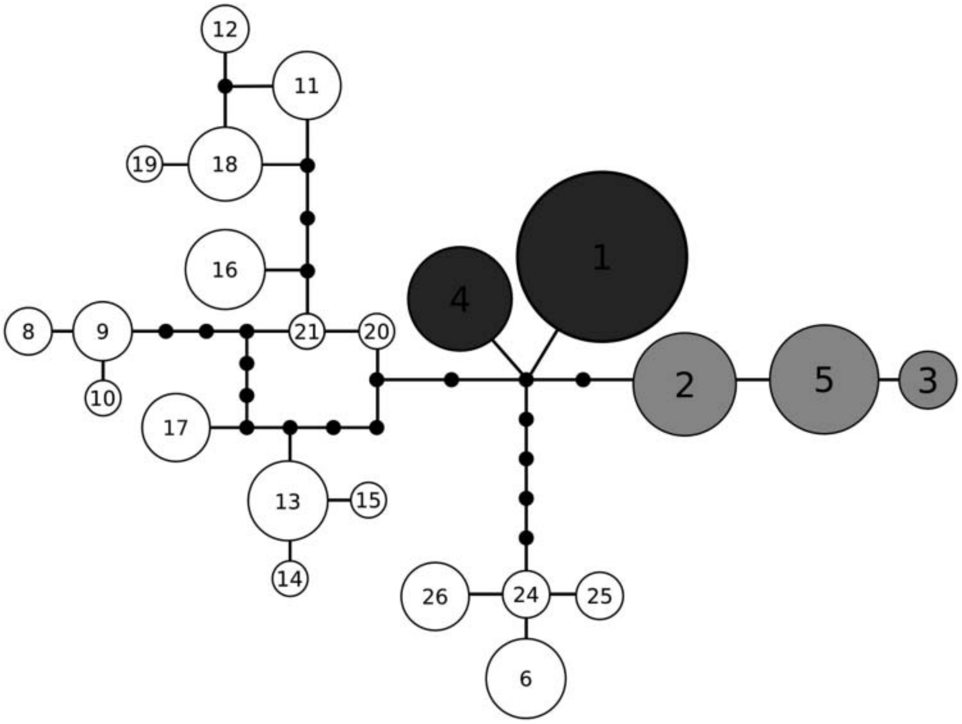

Fig. 5. MC1R statistical parsimony network of this nuclear gene for *D. praticola* sensu lato including outgroup species. Light grey represents the Balkan lineage and dark grey the Caucasian lineage. The circles in white correspond to *Darevskia* species that do not belong to the *praticola* group. Numbered circles correspond to unique haplotypes, as in Table 2, their size reflecting the number of individuals per haplotype. Small black dots represent the median vectors.

Table 3. Divergence times (tMRCA) for each split considered in the *BEAST species tree for *Darevskia praticola* sensu lato in millions of years.

Split	*BEAST height (95% interval)
Caucasus vs Balkans	2.500 ± [0.7540−5.120]
Caucasus Krasnodar-RU vs Caucasus Armenia-Iran	0.6574 ± [0.0727−1.557]

Armenia, and Iran and haplotype 4 is restricted to the Western Greater Caucasus. Haplotypes 2, 3, and 5 are found in the Balkans. Thus, Caucasus region haplotypes are more closely related to each other than to the Balkan haplotypes. On the other hand, both clades are more closely related to each other than to any other *Darevskia* species analysed here.

In the species tree reconstruction, the group assignment of individuals followed the ND4 tree topology (Fig. 4) and nuclear markers networks, concordant with geographic distribution.

Individual gene trees from the *BEAST analysis overall show the same pattern as the species tree. The RELN gene tree, however, placed haplotype 3 from the Caucasus closer to the Balkan haplotypes than to the remaining Caucasian haplotypes (Fig. 4).

The divergence time between the Balkan clade and the two Caucasian lineages is estimated at approximately 2.5 Ma (95% HPD interval 0.75−5.12 Ma) (Table 3). The divergence between the Greater Caucasus and Transcaucasia lineages is estimated at approximately 0.6 Ma (95% HPD interval 0.073−1.56 Ma). In the tree (Fig. 6), these lineages are identified as “Caucasus_RU” for the Greater Caucasus lineage and “Caucasus_ArmIran” for the Transcaucasia lineage.

Discussion

Amplification of a pseudogene for Cyt-*b*

The phylogenetic reconstruction based on Cyt-*b* is discordant with the phylogenetic reconstruction based on other loci (Fig. 2). The Cyt-*b* tree suggests *D. praticola* is not monophyletic and composed of two strongly diverged, non-sibling lineages, whereas the other three genes (gene trees from the *BEAST output, MC1R network and unpublished MC1R Bayesian tree) agree on the monophyly of *D. praticola*. This topological disagreement is particularly striking for Cyt-*b* and ND4 trees as both are mitochondrial genes and should be inherited as a single linkage block.

One of the two divergent Cyt-*b* clades includes all Balkan samples and a previously published sequence from the northwestern Caucasus (U88612) (Fu et al., 1997). This individual was collected in the vicinity of Sochi (43°35′N, 39°46′E) in the Western Greater Caucasus and this Cyt-*b* sequence was obtained with a different primer pair and PCR conditions than the ones used in our study (Fu et al., 1997). The other clade includes all our samples from the northwestern Caucasus and represented a highly divergent clade in the tree.

The divergence between the Balkan and Caucasian clades in the ND4 tree is much lower than the corresponding divergence in the Cyt-*b* tree (4.4% vs 12.5%), even though ND4 is typically a faster evolving gene. On the other hand, the ND4 divergence between the Caucasian and Balkan clades (4.4%) is concordant with the distance between the GenBank sequence (U88612) from the Caucasus and the Balkan clade in the Cyt-*b* tree (4.6%).

The pattern observed for Cyt-*b* is similar to that of confirmed pseudo-gene cases in lacertids (e.g. Podnar, Haring, Pinsker, & Mayer, 2007). We did not detect unusual amino-acid changes, stop codons, disturbed reading frame or double peaks in these sequences (results not shown). However, the low intralineal variation within the Cyt-*b* not monophyletic lineage, the discordant tree topology between different mtDNA markers and the different nucleotide content for the 3rd nucleotide codon position (data not shown) strongly suggest our Cyt-*b* sequences from the Caucasus represent a nuclear pseudo-gene (nuclear mitochondrial DNA or numt). Indeed, the study of numts can be extremely interesting from an evolutionary point of view and could even help to understand the organisms’ evolutionary history (e.g. Miraldo et al., 2012; Zevering, Moritz, Heideman, & Sturm, 1991). However, considering the phylogenetic objective of this study, the matter should be investigated independently and Cyt-*b* was excluded from further analysis.

Phylogenetic relationships within *Darevskia* and phylogeography of *D. praticola* sensu lato

Our results highlight the limitations of approaches based on single molecular markers. Most of the phylogenetic analyses of the genus *Darevskia* published to date are based on Cyt-*b* data (Murphy et al., 1996; Tarkhnishvilli 2012). Yet, our results show that commonly used, universal primers for Cyt-*b* may amplify numts in some taxa and lead, in turn, to erroneous species tree reconstruction.

The evolutionary history of *Darevskia* is complex and characterized by frequent hybridization events between different species (Danielyan et al., 2008; Darevsky, 1967). Some of these hybridizations are responsible for the origin of parthenogenetic taxa (Murphy et al., 1996; Tarkhnishvilli 2012). Therefore, a more thorough analysis of the relationships within *Darevskia* is needed to better understand the complexity of its evolutionary history. Given the peculiar reticulate evolution within this genus, an analysis with a wide selection of markers across the genome is needed in order to fully understand the hybridization patterns and intricate relationships within this genus.

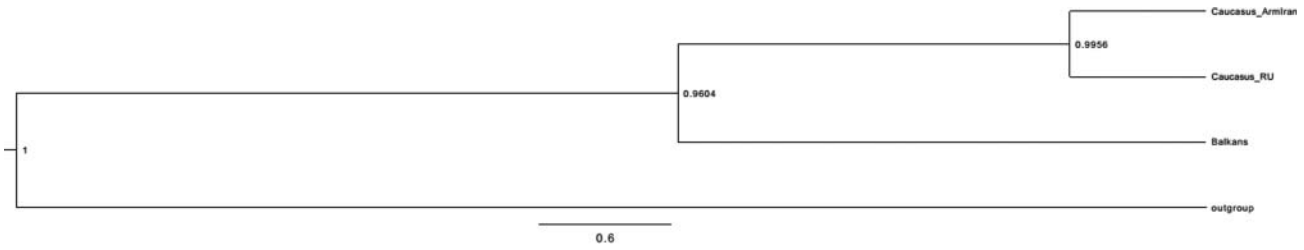

Fig. 6. Species tree recovered from *BEAST analysis of three genes (ND4, RELN, and MC1R). The traits used (“Caucasus”, “Balkans”, “Armenia and Iran” and “Russian Caucasus”) were selected according to the ND4 tree lineages and concordant with geographic distribution.

Regarding single marker analyses, the ND4 tree (Fig. 3) recovered three main lineages, and a basal division into a clade including all individuals from the Balkans (currently assigned to *D. praticola pontica*) and a clade including all individuals from the Caucasus. The Caucasus clade is further sub-divided into two sister lineages, the Transcaucasia and the Greater Caucasus individuals. The divergence between these two clades is approximately 5%. Within the Caucasus, the first lineage has the individuals from the western Greater Caucasus, which given its geographic origin are currently classified as *D. praticola pontica* (numbers 6, 7, and 8 in Fig. 1). The other has all the Transcaucasia individuals from the Talysh Mountains in Iran, currently classified as *D. praticola hyrcanica*, and a population from Armenia, classified as *D. praticola loriensis*. There is a slight geographic structure within the Transcaucasia lineage in this tree, even though this is not shared by the nuclear markers.

The MC1R network (Fig. 5) confirms the major clades found in the ND4 tree, separating the Balkan and the Caucasus individuals into two groups and supporting the geographic split. Also, these clades are closer to each other than to any of the other *Darevskia* analysed, reinforcing the monophyly of the *D. praticola* group. Apart from that, a MC1R tree made with the same samples used in this network grouped all *D. praticola* individuals in a well-supported monophyletic group (data not shown).

The geographic split between the Balkans and the Caucasus is also depicted by the RELN network (Fig. 4). However, some samples from the Caucasus (haplotype number 3) are closer to the Balkan individuals (13 substitution steps) than to the other Caucasus individuals (32 substitution steps). This could be explained by incomplete lineage sorting, frequent in nuclear markers even across species, but also by an overestimation of the gaps as a substitution step. This haplotype has the longest indel in the alignment, and that may have allowed for the overestimation of the distance between the Caucasus individuals. This is also shown in the RELN single tree resultant from the *BEAST analysis, which depicts one lineage that group samples from the different geographic regions. Nevertheless, the several indels may also have affected the way the sequences were sorted in the tree.

Our results provide the first phylogeographic analysis of *D. praticola* across its disjunct range. Both mitochondrial (ND4) and nuclear loci (MC1R and RELN) identify the presence of a strong and concordant phylogeographic structuring. Balkans and Caucasus do not share nuclear haplotypes and regional haplotypes are more closely related to each other than to haplotypes from the other region. Therefore, our data support long-term evolutionary independence of the *D. praticola* populations inhabiting the Balkans and Caucasus. Our results also depict divergence between samples from different areas within the Caucasus: two sister clades are found here, one with samples from the Greater Caucasus (Krasnodarskyi Kray, Russian Federation) and the other with the samples from Transcaucasia (Armenia, Azerbaijan-Iran). Moreover, a slight differentiation is depicted within Transcaucasia, where Armenian samples can be differentiated from the Iranian samples (from the Talysh Mountains). Overall, these results are not concordant with the current intraspecific taxonomy based on morphological characters of scalation and colouration (Ilgaz & Kumlutaş, 2005; Ljubisavljevic et al., 2006; Tuniyev et al., 2011) and suggest the need for intraspecific taxonomic revision of *D. praticola* that will take molecular data into account.

Evolutionary history

The divergence between the Balkan and the Caucasus lineages is estimated at approximately 2.50 Ma (95% HPD interval 0.75–5.12 Ma), at the end of the Pliocene. During the Pliocene, *D. praticola* must have been distributed along the Black Sea coast, from the Caucasus to the Balkans. At the end of the Pliocene (2.4 Ma), a major glaciation (Hays et al., 1976) possibly fragmented its distribution, and relict populations could have survived only in suitable habitat patches.

Given the uncertainty and debate around the palaeoclimatic events in the Black Sea region during the Pliocene–Pleistocene, it is hard to determine whether the north or south shore of the Black Sea was the most likely route of the *D. praticola* expansion.

In the early Pliocene the northern shore of the Black Sea was under shallow brackish water. The coast was intersected by branched river systems expanding over the major part of the western East European platform and the Black Sea basin was connected to the Caspian Sea basin (Webb & Bartlein, 1992). During the Middle Pliocene (3.6−2.5 Ma), the weather was hotter and wetter than today, and the major part of Eurasia was covered by deciduous forest (Willis, Kleczkowski, & Crowhurst, 1999). After that, during the Upper Pliocene (2.5−1.84 Ma), the temperature decreased and the forest area regressed.

At the end of the Pliocene (2.58 Ma), the Balkans and Asia Minor became connected (Kerey et al., 2004) and during subsequent Pleistocene glacial cycles the sea level fluctuated and the Black Sea connection to the Mediterranean Sea (via Marmara Sea) was frequently discontinued (or at least significantly restricted) (Popov et al., 2006). The aridification of the climate during the interglacials facilitated the vicariance between the Balkan and Caucasian forests, despite the land connections along the northern and southern Black Sea coast. Only during the late Pleistocene did the forests expand again (Willis et al., 1999), and the Black Sea level rose due to the melting of glaciers. At that time, the Late Pleistocene, the Bosphorus Strait was a narrow canyon not allowing a large flow of water from the Black Sea to the Marmara Sea, which led to the water accumulation and a rise in the Black Sea level (Esin & Esin, 2014). These vicariant events likely shaped the phylogeography of *D. praticola*. Similar palaeoclimatic scenarios have been proposed for lineage diversification in other reptile and amphibian species in this region (Ahmadzadeh et al., 2013a, 2013b, 2013c; Veith, Schmidtler, Kosuch, Baran, & Seitz, 2003; Wielstra, Baird, & Arntzen, 2013a). Also, this divergence time estimate overlaps with other co-distributed species and is likely to be associated with the Pliocene-Pleistocene transition (Ahmadzadeh et al., 2013b; Veith et al., 2003).

Pleistocene glaciation cycles facilitated genetic divergence in a variety of organisms due to range fragmentation in glacial refugia, whereas range expansion during interglacials likely allowed their admixture (Hewitt, 2000). The southern European peninsulas, i.e. the Iberian, Apennine, and Balkan Peninsulas (Salvi, Harris, Kaliontzopoulou, Carretero, & Pinho, 2013) as well as the Caucasus (Ursenbacher et al., 2006) played an important role as glacial refugia, providing environments of mild climatic conditions suitable for the survival of various organisms during colder periods. These southern refugia acted as cradles of diversity and speciation and provided the pioneers for interglacial re-colonization of Europe (Knowles, 2001).

Taxonomic implications

Several publications have suggested the existence of two distinct species within *D. praticola*. Using morphological characters, Tuniyev *et al*. (2011) proposed species status for *D. praticola* and *D. pontica*, assigning the lectotype for the latter from the northwestern Caucasus. They treated the Balkan populations as conspecific with *D. pontica* from the northwestern Caucasus. *Darevskia pontica* was distinguished from *D. praticola* based on the presence of a 3/3 arrangement of chin shields, longer head, distinct upper positioned masseteric plate in the temporal region (approaching toward tympanic plate and supratemporals), and tympanic plate separated from the supratemporals (Ljubisavljevic et al., 2006; Tuniyev et al., 2011). However, these authors conceded a degree of uncertainty regarding these characters in both taxa especially due to a putative overlap in these characters in the population inhabiting Stravropol Plateau, where individuals with 3/3 ("*D. pontica*") and with 2/2 ("*D. praticola*") chin shields are found sympatrically. Although we did not have access to samples from this region, the fact that our samples west and east from this area all fell within the Caucasian clade suggests that if there is any contact there it should be between its subclades, and not between the Caucasian and the Balkan clades. Ryabinina et al. (2011) had already proposed the existence of divergent Caucasian and Balkan clades, although the limited sampling and the type of markers used (RAPD and inter-MIR-PCR) do not allow a full comparison of these findings with our results.

The hypothesis proposed by Tuniyev et al. (2011) requires a recent dispersal between the Balkans and northwestern Caucasus. Our multilocus molecular results reject this hypothesis. Instead, they indicate a deep divergence between the Balkan and the Caucasus populations, dating back to the start of the Pleistocene. The northwestern Greater Caucasus lineage is much more closely related to the Transcaucasian lineage than to the Balkans clade. The northwestern Caucasus lineage includes *D. p. pontica* as well as part of the range of *D. p. praticola*. Because a lectotype for *D. praticola* has been designated from Gagry, northwestern Caucasus (Tuniyev et al., 2011), *D. p. pontica* should be considered a junior synonym of *D. p. praticola* and the name *D. praticola* should be applied to the Caucasus lineage. As suggested by Ljubisavljevic et al. (2006), the name *D. p. hungarica* first described by Sobolevsky in 1930 (Stugren 1961) with the type locality in the Transylvanian Alps, should be applied to the Balkan lineage.

Our results suggest that *D. p. hyrcanica* and *D. p. loriensis* represent two less divergent mtDNA lineages that share nuclear haplotypes. This would be concordant with their treatment as subspecies of *D. praticola*. This is partially consistent with phenotypic assignment of the Caucasian individuals to *D. praticola praticola* and the group formed by *D. praticola loriensis* (Armenia) and *D. praticola hyrcanica* (Talysh Mountains) (Fig. 3). The same subspecific pattern of diversification in the Caucasus has been recovered in other reptile and amphibian species

(Ahmadzadeh et al., 2013a, 2013b, 2013c; Wielstra et al., 2013a, 2013b), highlighting its role as a glacial refugium. Our results clearly indicate the presence of geographic structure of genetic diversity within the Caucasus which merits a more detailed analysis.

Authors' contributions

SF, MA, SD, JC-I, AK, DC, CC, PL, and MC performed the sample collection. MC was responsible for the funding for the molecular work. SF performed the analyses. SF and AV generated the molecular data. SF, SD, JH, and MC discussed the results. SF, SD, MA, and JH wrote the manuscript. All authors read, corrected and approved the final manuscript.

Acknowledgements

We thank S. Ferreira, S. Rocha and B. Wielstra for their valuable comments on the manuscript. N. Sillero, F. Jorge, A. Vardanyan, S. Ferreira, K. A. Matushkina, A. L. Timoshina, and E. Galoyan assisted with fieldwork. We further thank the anonymous reviewers, whose comments contributed for the improvement of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This research was partially funded by FCOMP-01-0124-FEDER-007062 FCT through the project PTDC/BIA-BEC/101256/2008, by the project "Biodiversity, Ecology and Global Change" co-financed by North Portugal Regional Operational Program 2007/2013 (ON.2 – O Novo Norte), under the National Strategic Reference Framework (NSRF), through the European Regional Development Fund (ERDF) and by the project "Preserving Armenian biodiversity: Joint Portuguese – Armenian programme for training in modern conservation biology" of Gulbenkian Foundation (Portugal). JCI was funded by Grant No.173025 "Evolution in heterogeneous environments: adaptation mechanisms, biomonitoring and biodiversity conservation" of Ministry of Education, Science and Technological Development of Republic of Serbia.

Supplemental data

Supplemental data for this article can be accessed http://dx.doi.org/10.1080/14772000.2015.1111267.

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Associate Editor: Mark Wilkinson