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RESEARCH ARTICLE

Phylogeography of the red-bellied lizard, *Darevskia parvula* in Turkey

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ABSTRACT

The groups of red-bellied lizards had a small distribution area in the Pontic zone. The several studies performed on these lizard groups are based on taxonomy and systematics. Although there were several taxonomic or systematic researches on some species of this group, the phylogeographical pattern and species disturbing boundaries of this group is still not clear. In the present study, we aimed to resolve the taxonomic and phylogenetic relationships of the red-bellied lizards in Turkey, based on two combined mitochondrial gene fragments and one protein-coding nuclear gene (*rag1*). Also, we evaluated ecological niches differentiations among red-bellied lizard groups. The mitochondrial DNA genes were found to be highly polymorphic in this group. One hundred and one variable nucleotide sites were detected on the combined gene sequences. According to phylogenetic trees based on the maximum likelihood (ML) and Bayesian inference (BI), the red-bellied lizards group have three species groups; *Darevskia parvula*, *D. adjarica* and unnamed *Darevskia* sp. (candidate species for *Darevskia* genus). This situation was supported by high bootstrap and posterior probability values in the trees of mitochondrial DNA gene fragments. However, no genetic variation was detected according to nuclear DNA (*rag1*) sequence. Because the species groups have no overlaps in terms of their ecological niches, ecological niche modelling (ENM) results revealed differences among the groups of *D. parvula*, *D. adjarica*, and unnamed *Darevskia* sp. Besides, we detected no geographical overlaps among three species groups, since there were geographical isolation zones among the species groups of red-bellied lizard.

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Introduction

The phylogeography is an important instrument to determine the genetic and geographic status of the populations of a species. Besides, studies based on phylogeographic events play a key role to connect evolutionary processes, including the comparative taxonomy affected the geological history, geomorphologic characters and climate changes of a study area (Daza et al., 2010; Burbrink et al., 2011; Turchetto-Zolet et al. 2013). According to the ecological knowledge, all of the species have a special niche in their living area (Van Valen 1976), and many of them have geographical isolation zones that play an important role in speciation (Wiens 2004). Isolation zones do not only distinguish between species but can also lead to differentiation of their ecological requirements. This phenomenon changes the ecological niches of isolated groups, it may reinforce speciation along the evolutionary time scale (Barraclough and Vogler 2000; Rundle et al. 2000; Turelli et al. 2001; Ogden and Thorpe 2002; Kozak and Wiens 2006), and in addition to it allows the formation of allopatry between groups. This event is defined as the appearance of natural barriers or physical limitations that divide the geographic range (Wiens 2004; Kozak and Wiens 2006). For this reason, geographic isolation can affect both phylogeny and ecological niches among a species group, and it may cause to differ from each other.

The red-bellied lizard, *Darevskia parvula* was first described by Lantz and Cyrén (1913) from Artvin. The type specimens of the species were collected from Artvin (Çoruh Valley), Borçka and Ardanuç (Lantz and Cyrén 1913). Because the *D. parvula* is distributed in the Pontic region (eastern Black Sea, north of Eastern Anatolia and west and southwest Georgia) in the world (Darevsky 1967; Darevsky and Eiselt 1980; Sindaco et al. 2000), it is endemic to Pontic region. In this distribution area, the species have two distinct morphological subspecies as *D. p. parvula* and *D. p. adjarica* (Darevsky and Eiselt 1980). *Darevskia parvula parvula* is distributed inland of Artvin, Ardahan, Kars, and Erzurum and *D. p. adjarica* is distributed Trabzon, Rize and cost of Artvin (Darevsky and Eiselt 1980).

Many studies were formerly carried out to designate distribution, morphology and taxonomy of the species (Bodenheimer 1944; Darevsky 1967, Clark and Clark 1973, Başoğlu and Baran 1977; Darevsky and Eiselt 1980; Baran et al. 1997; Baran and Atatür 1998; Franzen 1999, 2000; Kutrup 2001; Baran et al. 2004; Ilgaz 2009). One of these studies was on the taxonomy and description of subspecies *D. p. adjarica* (Darevsky and Eiselt 1980). *D. p. adjarica* distinguished from the nominate subspecies *D. p. parvula* with the several morphological characteristics (pudgy body shape, lower number of supratemporalia, longer 1st supratemporal plate, different shape of the 6th submaxillary plate, existing

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darkness band on the dorso-lateral sides, more brown-black colour on dorsal region and more reddish ventral region in the adult individuals) according to the study of Darevsky and Eiselt (1980). Also, Darevsky and Eiselt (1980) reported *D. p. adjarica* is distributed between Trabzon and coastal Artvin and *D. p. parvula* is distributed in other remain inlands in Turkey. Contrary to Darevsky and Eiselt (1980), Ilgaz (2009) stated that Ardahan specimens (inland populations) were to be morphological subspecies of *D. p. adjarica*.

A recent study was published about morphology, osteology and the genetic reevaluating taxonomic status of *D. parvula* (Arribas et al. 2018). In this study, the authors concluded that two subspecies of *D. parvula* were distinct species as *D. parvula* and *D. adjarica*. However, this study did not give much information about the phylogeography of the *D. parvula*. The authors used 11 specimens in the genetic studies from three localities while they used 213 specimens in the morphological studies from ten localities in Turkey. Although their study is morphologically adequate, however, 11 specimens from 3 localities were not adequate for the phylogeography of the species. Because of this reason, the present, more comprehensive, study played an important role to the well-performed phylogeny of the *Darevskia parvula* in Turkey.

As can be seen from the literature, the number of localities and lizard samples for molecular data of *D. parvula* from Turkey was quite inadequate. With this way, the present study was purposed to:

i. reevaluate the taxonomy of the *D. parvula* using three molecular markers (partial sequences alignments of mitochondrial 16S rRNA and *cytb* genes and the nuclear *rag1* gene),
ii. elucidate the phylogeny in more detail using more samples (86 specimens) from more localities (33 localities),
iii. contribute to the determination of the geographical conditions that cause the formation of phylogenetic relationships of these lizards,
iv. investigate the genetic differences among individuals belonging to populations in different geographical and ecologically diverse environments such as different altitude, temperature and humidity,
v. identify potential lineages that can occur during the evolutionary process,
vi. and give information about the differences in ecological niches among red-bellied lizard populations in Turkey.

Materials and methods

Collection of the sample

During the field studies, a total of 86 specimens collected from 33 different localities in Turkey between 2014 and 2017 for the molecular studies (Figure 1) (Table 1). For each lizard, the longest finger of the hind limb was clipped and preserved in 96% ethanol. After registration and toe-clipping, all lizards were released back into their natural habitats. The animals were treated in accordance with the guidelines of the local ethics committee (KTÜ. 53488718-704 2015/14).

DNA extraction and PCR amplifications

The clipped toes obtained from lizards were stored in 96% ethanol. Later, the toes were treated with 180 µl ATL, 20 µl proteinase K and 4 µl RNAse in 2 ml Eppendorf tubes overnight at 56 °C. Total genomic DNA of each specimen was

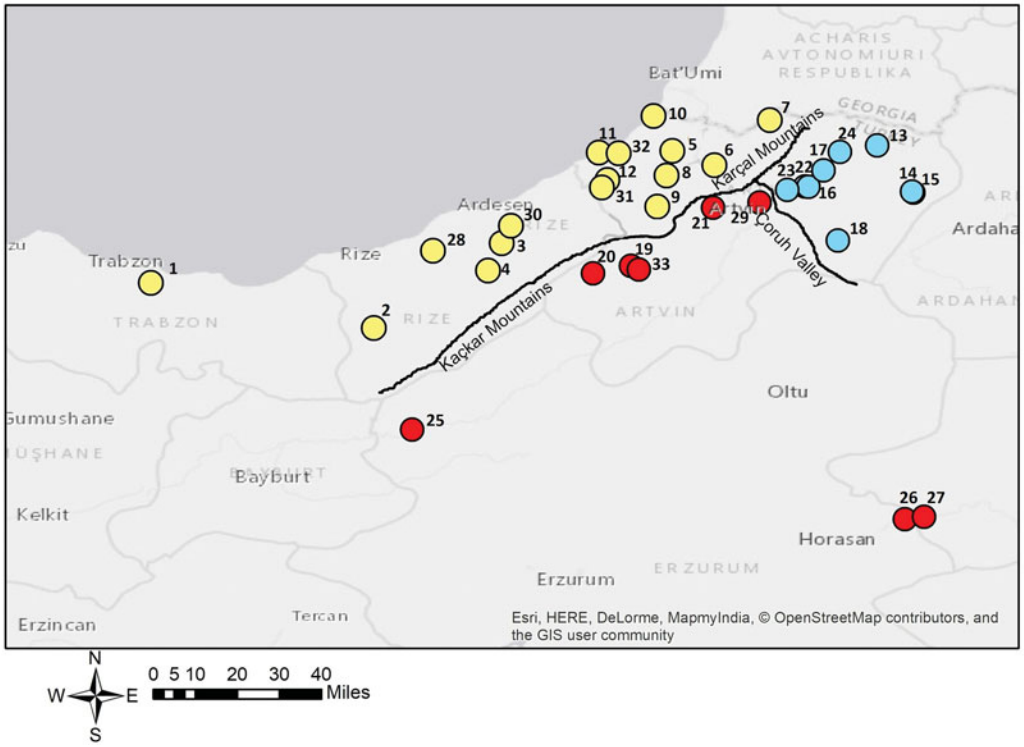

Figure 1. Locality records for red-bellied species group. (blue: *D. parvula*, yellow: *D. adjarica*, red: unnamed *Darevskia* sp.). Colours also represent mitochondrial haplogroups. Locality numbers were given in Table 1. Black line showed the isolation zone among three species groups.

Table 1. Sample localities, haplotype group and number of individuals which were used in phylogenetic analysis and GenBank accession numbers of haplotypes (*n*: number of samples).

No	Locality	Abbreviation	*n*	Haplotypes	Accession numbers		
					cytb	16S rRNA	*rag1*
1	Yomra	Par 1-2	2	Par 1	MK092669	MK092689	MK092651
2	İkizdere	Par 3-5	3	Par 3	MK092670	MK092690	MK092652
3	Çamlıhemşin	Par 6-8	3	Par 4	–	MK092691	MK092653
4	Zilkale	Par 9-11	3	Par 5	–	MK092692	–
5	Çifteköprü	Par 12-14	3	Par 6	MK092671	–	–
6	İbrikli Village	Par 15-17	3	Par 9	–	–	MK092654
7	Camili	Par 18-19	2	Par 12	MK092672	MK092693	MK092655
8	Murgul	Par 20-21	2	Par 13	–	MK092694	MK092656
9	Kabaca Village	Par 22	1	Par 15	–	MK092695	–
10	Kemalpaşa	Par 23-25	3	Par 18	MK092673	MK092696	MK092657
11	Arhavi	Par 26-28	3	Par 19	–	MK092697	–
12	Çiftekemer Köprü-Ortacalar	Par 29-31	3	Par 20	–	–	MK092658
13	Meydancık-Erikli Village	Par 32-34	3	Par 23	MK092674	–	MK092659
14	Sahara National Park	Par 35-37	3	Par 27	–	MK092699	–
15	İzzetkışla	Par 38	1	Par 28	–	MK092700	–
16	Soğuksu	Par 39	1	Par 29	–	–	MK092660
17	Maden Köyü	Par 40-42	3	Par 32	MK092675	MK092701	–
18	Geçitli Village	Par 43-45	3	Par 35	MK092676	MK092702	–
19	Barhal Valley	Par 46-48	3	Par 38	–	–	MK092661
20	Barhal ValleyAltıparmak Mountains	Par 49-51	3	Par 39	MK092676	–	–
21	Hatila Valley	Par 52-54	3	Par 41	–	MK092703	–
22	Pırnallı Village	Par 55-57	3	Par 43	MK092677	–	–
23	Ortaköy Village	Par 58-60	3	Par 45	–	–	MK092662
24	Yanıklı Village	Par 61-63	3	Par 46	MK092678	MK092704	–
25	Pazaryolu	Par 64-66	3	Par 47	MK092679	MK092705	–
26	Horasan	Par 67-69	3	Par 48	–	MK092705	–
27	Sarıkamış	Par 70-72	3	Par 50	–	MK092706	–
28	Çayeli	Par 73-75	3	Par 52	MK092680	MK092707	MK092663
29	Salkımlı Köyü	Par 76-78	3	Par 55	MK092681	–	–
30	between Ardeşen-Çamlıhemşin	Par 79-81	3	Par 57	–	MK092708	–
31	Kamilet Valley	Par 82-84	3	Par 58	–	–	MK092664
32	Yeşilköy Village	Par 85	1	Par 59	–	MK092709	–
33	Sarıgöl Village	Par 86	1	Par 62	–	–	MK092665
				Par 64	MK092682	MK092710	–
				Par 65	MK092683	MK092711	–
				Par 66	–-	MK092711	–
				Par 67	MK092684	MK092712	MK092666
				Par 70	MK092685	–	–
				Par 71	–	MK092713	–
				Par 73	MK092686	MK092714	–
				Par 74	–	MK092715	–
				Par 75	–	MK092716	–
				Par 76	MK092687	MK092716	–
				Par 77	–	MK092717	–
				Par 79	–	–	MK092667
				Par 80	MK092688	–	–
				Par 81	MK092686	–	–
				Par 82	–	–	MK092668
				Par 85	–	MK092718	–

extracted using the NucleoSpin® tissue isolation kit following Manufacturer's instructions.

A total of 537–539 base-pair-fragment of the 16S rRNA gene (for 86 specimens) and a total of 511–513 base-pair-fragment of the *cytb* gene (for 65 specimens) were amplified from mitochondrial DNA using 16SarL and 16SbrH (Palumbi et al. 1991) and, L15369 and H15915 (Fu 2000) primers, respectively, and a total of 822 base-pair-fragment of the *rag1* gene (86 specimens) were amplified from nuclear DNA using RAG-fo and RAG-re primers (Mayer and Pavlicev 2007). Accession numbers of all haplotype sequences were given in Table 1. Each 16S rRNA gene amplification involved an initial incubation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, the appropriate annealing temperature at 52 °C for 30 s, elongation temperature at 72 °C for 1 min and final extension temperature at 72 °C for 8 min. PCR amplifications for 16S rRNA were conducted as described by Guo et al. (2011). PCRs were performed in total volumes of 50 µl with 25 µL 2x One Taq® PCR, 1 µl F primer, 1 µl R primer, 21.5 µl ddH_2O and 1.5 µl of genomic DNA as a template. Each *cytb* gene amplification involved an initial incubation at 93 °C for 3 min, followed by 30 cycles at 93 °C for 60 s, the appropriate annealing temperature at 53 °C for 60 s, elongation temperature at 69 °C for 2 min and final extension temperature at 70 °C for 10 min. PCR amplifications for cytb were conducted as described by Gabelaia et al. (2015). Each *rag1* gene amplification involved an initial incubation at 94 °C for 2 min, followed by 35 cycles at 95 °C for 10 s, the appropriate annealing temperature at 52 °C for 15 s, elongation temperature at 72 °C for 50 s and final extension

temperature at 72 °C for 7 min. PCR amplifications for *rag1* were conducted as described by Mayer and Pavlicev (2007). Amplified DNA segments were purified and sequenced by Macrogen Corporation in Netherlands

Sequence alignment and phylogenetic analyses

All nucleotide sequences of the genes (16S rRNA, *cyt b* and *rag1*) used in the molecular analyses were aligned using BioEdit 7.0.5.3 (Thompson et al. 1997) program. Before finding haplotypes, mitochondrial genes (16S rRNA and *cyt b*) were combined, the suitability for the combination of all the sequences of two genes was confirmed. Haplotypes of the combined mitochondrial and nuclear gene were found using by TCS 1.21 (Clement et al. 2000) program. The best-fit substitution model was determined with MrModeltest v. 2.3 (Nylander 2004) and the best model was chosen according to the lowest AIC (Akaike's information criteria) degree (Akaike 1974) for each gene. Phylogenetic analyses were based on the combined genes (16S rRNA and *cytb*) and nuclear gene (*rag1*) separately. We conducted multiple complementary methods of data analysis, such as maximum likelihood (ML) and Bayesian inference (BI) phylogenetic approaches using MEGA 7.0 v (Kumar et al. 2016) for ML, and MrBayes 3.2.6 (Ronquist et al. 2011) for BI. Maximum likelihood (ML) analyses were carried out using a heuristic search method (10,000 random addition replicates tree-bisection-reconnection, TBR, branch swapping) and bootstrap analyses with 1000 replications ML (Felsenstein 1985) were applied. Transitions and transversions were equally weighted, and gaps were treated as missing data. In the BI analysis, the following settings were conducted: number of Markov Chain Monte Carlo (MCMC) generations = ten million; sampling frequency = 100; burn-in = 25%. The burn-in size was determined by checking convergence of –log likelihood (-ln L) using MrBayes 3.2.6 (Ronquist et al. 2011). ML trees were evaluated using bootstrap analyses with 1000 replicates and statistical support of the resultant BI trees was determined based on Bayesian posterior probability (BPP). Best fit nucleotide substitution models were determined for each gene region with MEGA 7.0 v (Kumar et al. 2016) for ML and BI analyses based on Akaike's information criteria (AIC). We *a priori* regarded tree nodes with bootstrap values (BS) 70% or greater as sufficiently resolved (Huelsenbeck and Hillis 1993), and those between 50 and 70% as tendencies. In the BI analysis, we considered nodes with a BPP of 95% or greater as significant (Leachè and Reeder 2002). Uncorrected pairwise sequence divergences for each gene (16S rRNA, *cytb* and *rag1*) were calculated using MEGA 7.0 v (Kumar et al. 2016). DQ494823.1 *Lacerta agilis* (Wan et al. 2007) and AF206182.1 *Darevskia lindholmi* (Fu 2000) sequences for 16S rRNA gene, AM087227.1 *Lacerta agilis* (Böhme et al. 2006) and AF206177.1 *Darevskia lindholmi* (Fu 2000) sequences for *cytb* gene and EF632236.1 *Parvilacerta parva* (Mayer and Pavlicev 2007) and EF632212.1 *Darevskia valentini* (Mayer and Pavlicev 2007) sequences for *rag1* gene were obtained from Gene Bank for out groups in the phylogenetic analyses.

Geographic discrimination and ecological niche overlap analyzing

A total of 81 distributional records were collected from field studies (48 localities) and literature (33 localities) (Clark and Clark 1973; Darevsky and Eiselt 1980; Franzen 1990; Mulder 1995; Franzen 1999; Baran et al. 1997; Franzen 2000; Baran et al. 2004; Ilgaz 2009; Bülbül et al. 2016; Arribas et al. 2018). Forty-two of these localities are represented with *D. p. adjarica*, 19 of them are represented with *D. p. parvula* and the others are represented with new genetical clade (this discrimination was done according to phylogenetic trees of the mitochondrial DNA haplotypes and these mitochondrial groups did not overlap geographically).

Nineteen bioclimatic variables were downloaded from Global Climate Data to construct species distribution modelling (Hijmans et al. 2005; available at www.worldclim.org). These data were generated from global esri grids in the highest resolution [30″ (∼1 km)] for current conditions (∼1950–2000). Each bioclimatic variable was limited the land border of Turkey using by Arc Toolbox (extract by mask) in ArcGIS ver. 10.3 software. In order to eliminate the negative effect may be resulted from other environmental variables, Pearson correlation coefficient ($0.75 < r << -0.75$) was performed using ENMTools 1.4 (Warren et al. 2010) for all variables, and highly correlated variables were left out of analysis for the distribution modelling of *D. parvula*.

After the correlation analysis, Maxent 3.3.3k (Phillips et al. 2006) software was used to perform species distribution modelling. To develop the model, 81 occurrence data based on literature and new locality records were used. Twenty-five percent of the occurrence data were set aside as test points, and 10.000 background points were used to determine the distribution. Additionally, the regularization multiplier = 0.5, maximum iterations = 500, and convergence threshold = 10–5 were chosen in Maxent. In order to test the variable importance, the Jackknife test of variable importance was chosen in Maxent, and the model was run as ten replicates. The result of the receiver operating characteristic (ROC) curve is important for model sensitization and the value of the area under the curve (AUC) closest to 1 indicated the best model performance. A value near 0.5 suggests that the result is not better than random (Raes and ter Steege 2007; Gallien et al. 2012).

The identity test is used to test habitat suitability scores for three mitochondrial groups to assess significant niche differences generated by ENM (Warren et al. 2010). ENMTools was employed to calculate the niche overlap test to examine niche divergence between species. Schoener's D (Warren et al. 2008) and Hellinger's-based I (Schoener 1968) are two indices for niche identity and were calculated based on the habitat suitability comparison from ENM. Schoener's D calculates the suitable range for a given species based on probability distributions for inhabiting particular regions (cells), calculating niche overlap based upon species abundance in those locations. Hellinger's-based I is based purely on probability distributions without the assumptions of Schoener's D. (Warren et al. 2010). Both indices range from 0 (complete divergence/no overlap) to 1 (high similarity/complete

overlap). Background tests were performed to evaluate whether the ecological niches of three mitochondrial groups are different from each other beyond expected differences based upon the environmental conditions that they require (Warren et al. 2008). We compared the niche models of potential habitat for each species with a series of 100 pseudoreplicate models generated using data from the other (Warren et al. 2008). The Schoener's D and Hellinger's-based I of the true calculated niche were compared to the null distribution of 100 replicates (Warren et al. 2008).

Results

Phylogeny and sequence variation

A total of 537–539 base pairs of 16S rRNA and 511–513 base pairs of *cytb* gene were obtained from *D. parvula* individuals, and they were combined. At the end of the combination, a total of 1048–1052 homologous base pairs of the combined mitochondrial DNA (16S rRNA and *cytb* genes) were obtained from 65 individuals and a total of 822 homologous base pairs of the *rag1* genes were obtained 86 individuals. Totally, 40 haplotypes for combined mitochondrial DNA and 20 haplotypes for *rag1* gene were found. Mitochondrial haplotypes show four separate groups (Figure 2). One of them is *D. p. parvula* including eight haplotypes. The other haplotype group is *D. p. adjarica* including nineteen haplotypes, and the others are new genetical clade (unnamed *Darevskia* sp.) including twelve and one haplotypes, respectively. However, nuclear haplotypes did not support any separate groups (Figure 2). The studied fragments of combined mitochondrial genes and nuclear gene had 101 and 48 variable positions, respectively. Sixty-two positions of the partial mitochondrial genomes had differences in the haplotype groups, and these differences showed geographical discrimination for mitochondrial haplotypes (Figure 1). Other variations of the mitochondrial genes were individual differences of haplotypes. Nuclear gene did not show any differences among haplotype groups. According to model test results, the best-fit substitution model was chosen as GTR + G + I (Rodríguez et al. 1990) for

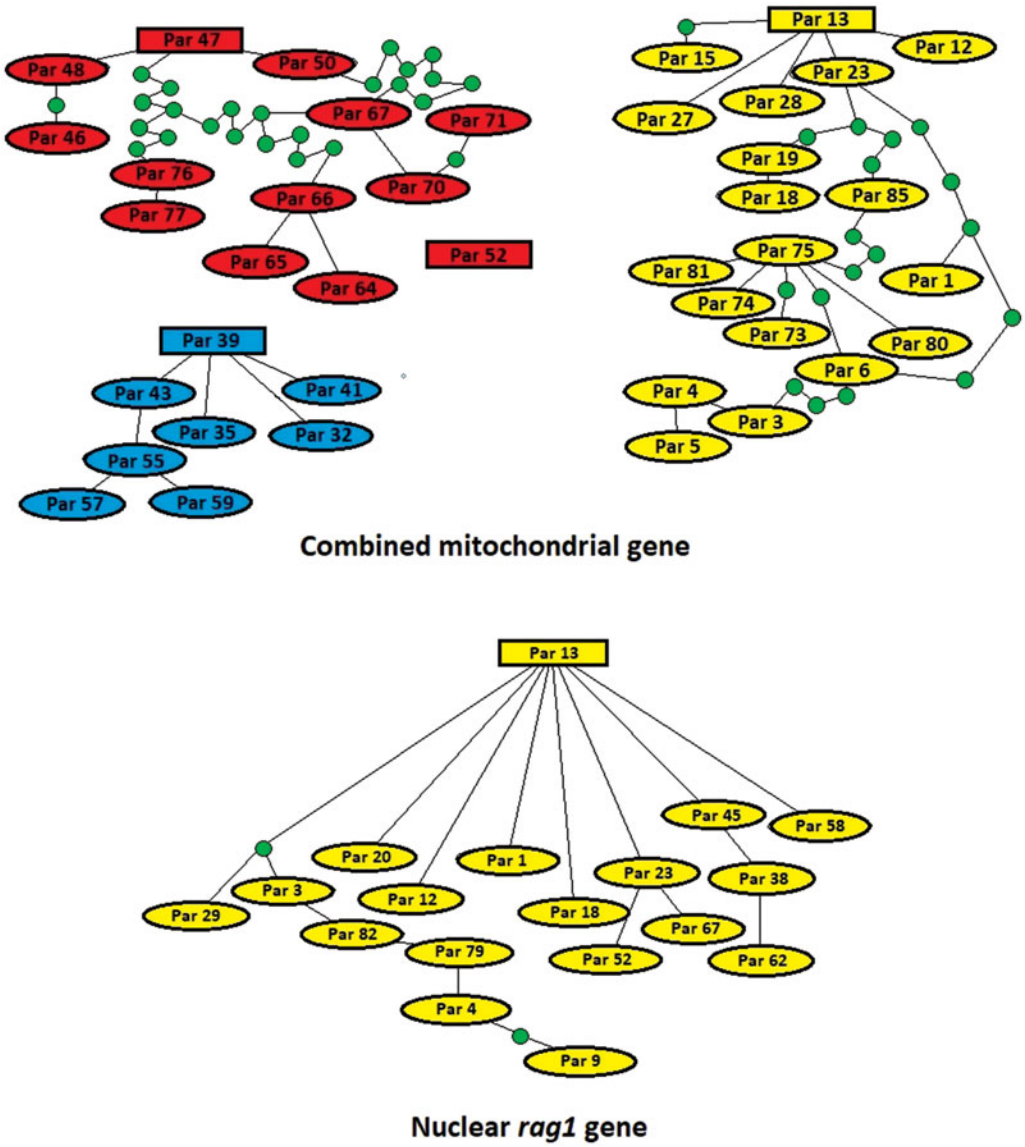

Figure 2. Haplotype networks of combined mitochondrial and nuclear DNA. Haplotype numbers were indicated in Table 1.

combined mitochondrial genes and HKY + G + I (Hasegawa et al. 1985) for *rag1* gene.

The ML tree of the mitochondrial haplotypes is shown in Figure 3. The rooted tree is divided into two well-supported clades with 100 bootstraps. One of these clades represented *D. p. parvula* and *D. p. adjarica* and the other clade is genetically new for *D. parvula* species. The genetical new clade was divided into two subclades. One of them included 12 haplotypes and the other included one haplotype. The locality of subclade with one haplotype for new genetical clade is Hatila valley (Par 52). Hatila valley was similar geographical location with some haplotypes for new genetical clade. Because of this, it does not show geographical discrimination from some others. Also, mitochondrial ML tree showed geographical discrimination. Kaçkar and Karçal mountain ranges separated the *D. p. adjarica* subclade from the others (*D. p. parvula* and new genetical clade) and the mountain series supplied the isolation region for *D. p. adjarica*. Besides, *D. p. parvula* is divided from new genetical clade with the Çoruh river arms passed on the Ardanuç and Şavşat detour (Figure 1). Bayesian inference was full concordance with the ML tree for mitochondrial genes (Figure 3). The posterior probabilities (1.00) showed well-supported points with the ML tree.

The ML tree of the haplotypes of *rag1* gene is shown in Figure 4. The ML tree of nuclear gene did not support with the ML tree of combined mitochondrial genes. All of the haplotypes were represented with the one clade for *rag*1 gene. Bayesian inference was full concordance with the ML tree for nuclear gene (Figure 4). The posterior probabilities were lower 0.95 and it did not support discrimination of the haplotypes.

Mitochondrial DNA showed higher genetical distance for 40 haplotypes with 1048–1052 base pairs while genetic diversity of the nuclear DNA was very lower for 20 haplotypes with 822 base pairs. P-distance ranged from 0.1 to 6.8% for combined mitochondrial genes, while it ranged from 0.1 to 1.0% for *rag1* gene. P-distance ranged from 1.8 to 2.8% between *D. p. parvula* and *D. p. adjarica*, while it ranged from 6.2 to 6.8% between *D. p. parvula* and new genetical clade. Besides, p-distance ranged from 5.4 to 6.8% between *D. p. adjarica* and new genetic clade. Separately, p-distance for 16S rRNA gene ranged from 0.8 to 1.8% between *D. p. parvula* and *D. p. adjarica*, while it ranged from 1.5 to 3.6% between *D. p. parvula* and new genetical clade. Besides, p-distance of 16S rRNA ranged from 1.5 to 4.3% between *D. p. adjarica* and new genetic clade and p-distance for *cytb* gene ranged from 2.8 to 4.2% between *D. p. parvula* and *D. p. adjarica*, while it ranged from 10.3 to 12.5% between *D. p. parvula* and new genetical clade. Besides, p-distance of 16S rRNA ranged from 8.1 to 11.6% between *D. p. adjarica* and new genetic clade (Table 2).

Ecological niche divergence and identity test

We chose a total of 10 variable to use ecological niche modelling for three mitochondrial haplogroups, and these

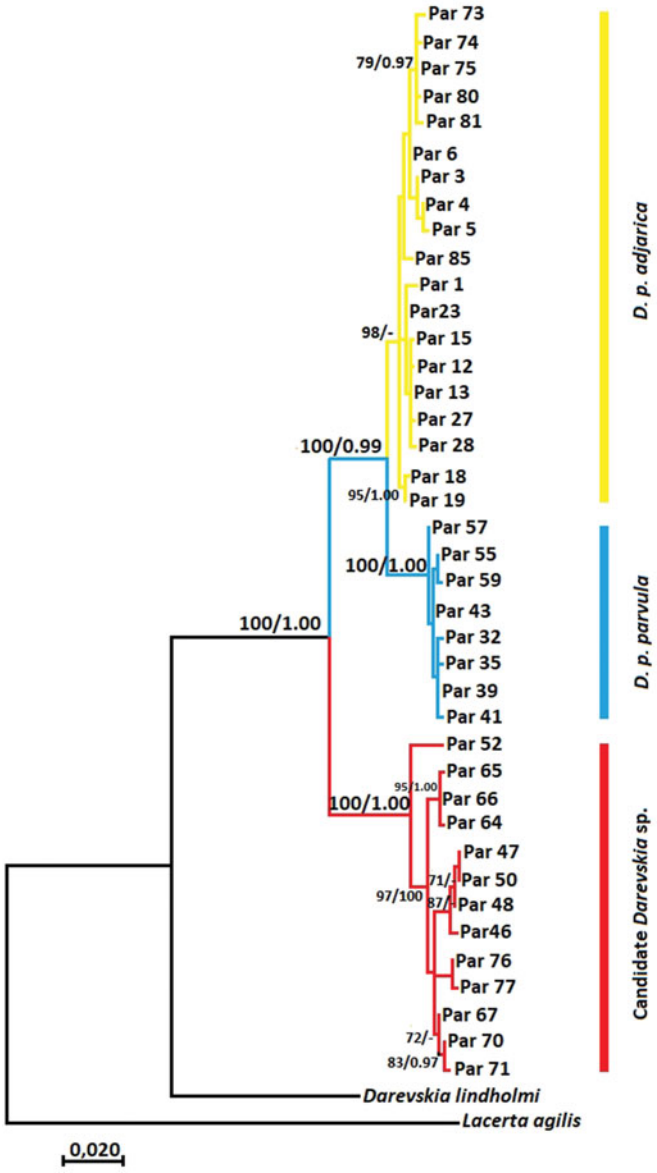

Figure 3. Phylogenetic tree based on combined mitochondrial DNA data set. Bootstrap and posterior probability values given by ML/BI.

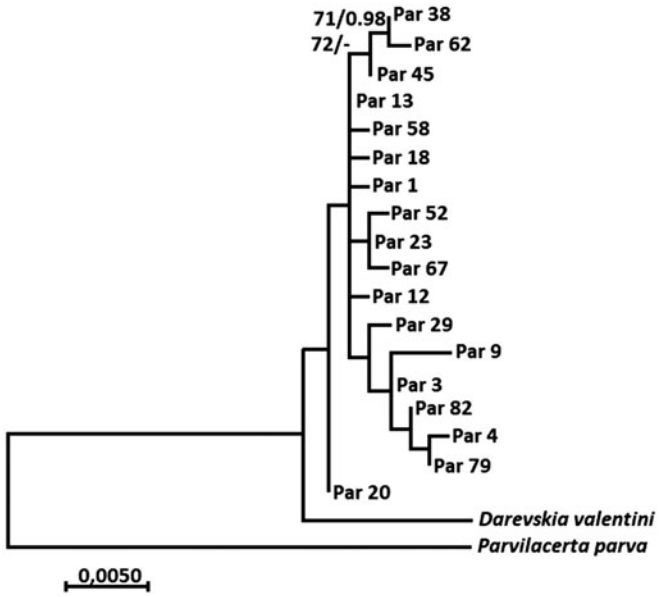

Figure 4. Phylogenetic tree based on nuclear DNA data set. Bootstrap and posterior probability values given by ML/BI.

variables were used in the present study. Bio-4 (Temperature Seasonality (standard deviation *100)), Bio-9 (Mean Temperature of Driest Quarter), Bio-15 (Precipitation Seasonality (Coefficient of Variation)), Bio-18 (Precipitation of Warmest Quarter), altitude and slope variable were chosen for *D. p. parvula*, Bio-4 (Temperature Seasonality (standard deviation *100)), Bio-5 (Max Temperature of Warmest Month), Bio-14 (Precipitation of Driest Month), Bio-15 (Precipitation Seasonality (Coefficient of Variation)), altitude and slope variable were chosen for *D. p. adjarica* and, Bio-7 (Temperature Annual Range (BIO5-BIO6)), Bio-9 (Mean Temperature of Driest Quarter), Bio-14 (Precipitation of Driest Month), Bio-15 (Precipitation Seasonality (Coefficient of Variation)), Bio-19 (Precipitation of Coldest Quarter), altitude and slope variable were chosen for new genetical clade. The contributions of the variables were given in Table 3.

Geographical record of three mitochondrial groups has no overlap in their distribution ranges (Figure 5). Ecological models for three mitochondrial groups confirmed the occurrence of suitable areas for their presence in Turkey (Figure 5). Estimated training AUC values ± standard deviation (SD), 0.998 ± 0.001 for *D. p. parvula*, 0.992 ± 0.006 for *D. p. adjarica* and 0.986 ± 0.012 for new genetic clade of mitogenome indicated very good model results in comparison with random background points. The presence of the habitat suitability for the *D. p. parvula* was predicted to be in eastern Artvin including Şavşat and Ardanuç, the predicted habitat suitability presence for *D. p. adjarica* was shown in the costal Black sea including Trabzon, Rize and Artvin, and the presence of habitat suitability for new genetic clade of mitochondrial DNA was predicted to be south of the Black sea region and east Anatolian region including Erzurum Kars and Artvin province (Figure 5).

Table 2. Genetic distances among red-bellied lizard groups indicated by mitochondrial gene region.

16S rRNA	*D. p. parvula*	*D. p. adjarica*	*D. p.* ssp.
1 *D. p. parvula*	–		
2 *D. p. adjarica*	0.8–1.8%	–	
3 *Darevskia* sp.	1.5–3.6%	1.5–4.3%	–
cyt b	1	2	3
1 *D. p. parvula*	–		
2 *D. p. adjarica*	2.8–4.2%	–	
3 *Darevskia* sp.	10.3–12.5%	8.1–11.6%	–
16S rRNA+ *cyt b*	1	2	3
1 *D. p. parvula*	–		
2 *D. p. adjarica*	1.8–2.8%	–	
3 *Darevskia* ssp.	6.2–6.8%	5.4–6.8%	–

Table 3. Level contribution of variables used in Maxent model for three species groups.

No	Variables	*D. p. parvula* Contribution (%)	*D. p. adjarica* Contribution (%)	*Darevskia* sp. Contribution (%)
1	Bio-15	34.9	6.1	25.3
2	Bio-18	32.4	–	–
3	Bio-4	14.9	1.2	–
4	Bio-9	10.6	–	3.8
5	Bio-14	–	87.7	35.8
6	Bio-5	–	1.4	–
7	Bio-7	–	–	18.9
8	Bio-19	–	–	6.3
9	Altitude	4.4	1.3	3.6
10	Slope	2.9	2.4	6.4

Ecological niche modelling results showed that there is no niche overlap among three mitochondrial genome group (Hellinger's-based I = 0.45 and Schoener's D = 0.23 for *D. p. parvula*/D. p. *adjarica*, I = 0.44 and D = 0.27 for *D. p. adjarica*/new genetic clade and I = 0.50 and D = 0.26 for *D. p. parvula*/new genetic clade). The identity test indicated that our null hypothesis of niche overlap among three mitochondrial species groups was rejected and overlap among the three mitochondrial groups was significantly different (*t*-test, df = 99, $p < .05$). The model indicated that estimated niche models for *D. p. parvula*/*D. p. adjarica* (D_{H0} = 0.70 ± 0.06 vs. D_{H1} = 0.23 and I_{H0} = 0.91 ± 0.04 vs. I_{H1} = 0.45), *D. p. adjarica*/new genetic clade (D_{H0} = 0.73 ± 0.06 vs. D_{H1} = 0.27 and I_{H0} = 0.91 ± 0.04 vs. I_{H1} = 0.44) and *D. p. parvula*/new genetic clade (D_{H0} = 0.73 ± 0.07 vs. D_{H1}

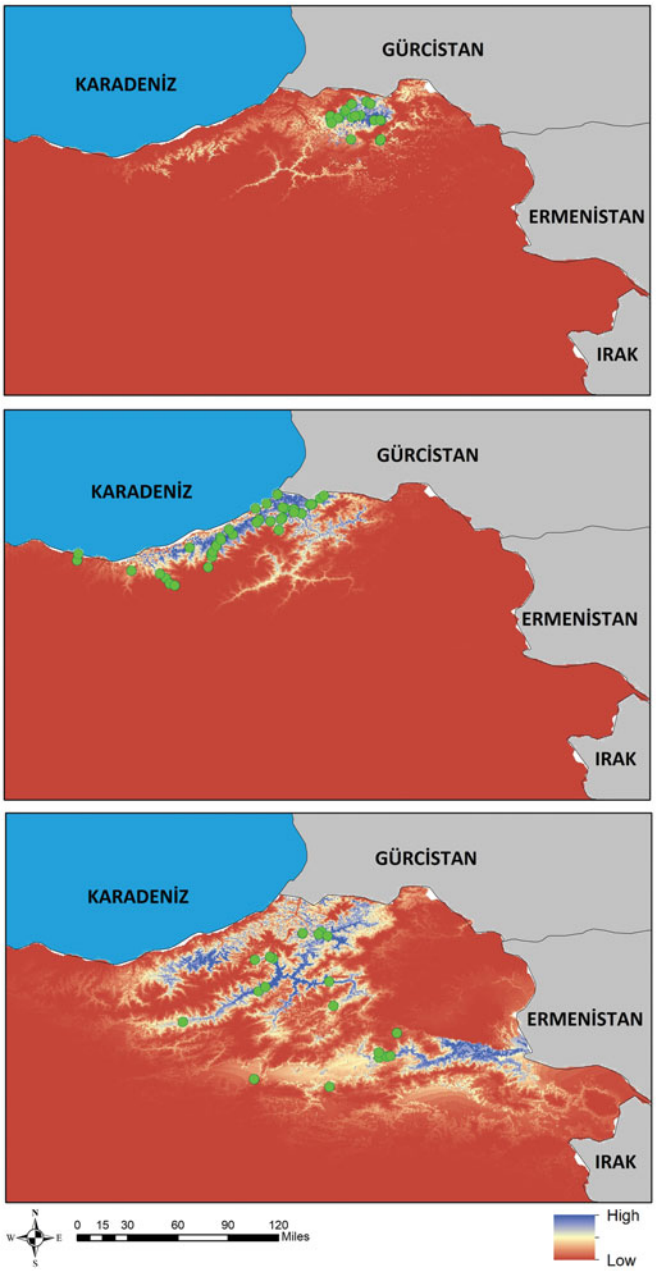

Figure 5. Habitat suitability for *D. parvula* (A), *D. adjarica* (B) and unnamed *Darevskia* sp. Three main colours show habitat suitability in the map as mentioned in the map legend. Red represents suitability of less than 0.52, white represents suitability between 0.52 and 0.70, and blue colour represents suitability greater than 0.70.

= 0.26 and $I_{H0} = 0.93 \pm 0.04$ vs. $I_{H1} = 0.50$) were completely separate and significantly distinct (Figure 6). The identity test indicated overlap among the three groups was significantly different. The model indicated that estimated niche models for three mitochondrial groups were completely separated and significantly distinct (Figure 6). According to niche results, because these three mitochondrial groups have different ecological niche, it can say that these groups will be separate species.

Discussion

The topologies of the phylogenetic trees and genetic variations of the combined mitochondrial genes in the present study showed three species group for red-bellied lizard. These species include *D. parvula*, *D. adjarica,* and new genetical clade, a new cryptic species (unnamed *Darevkia* sp.). According to a recent study based on external morphology, osteology and molecular, Arribas et al. (2018) stated that *D. parvula* and *D. adjarica* were different species as indicated the present study. However, they did not mention a cryptic diversity in the *D. parvula* group. They only focused on that subspecies *D. p. adjarica* was different from *D. p. parvula* in the species level. Furthermore, the phylogenetic tree generated for *cytb* gene in their study did not show clear separation. According to their phylogenetic result, the tree topology separated two clade and Arhavi population was also present within these two lineages.

The genetic distance between *D. parvula* and *D. adjarica* was found 14.4% using *cytb* gene with 306 base pair sequence in the study of Arribas et al. (2018). Contrary to this, our genetic distance results showed lower variability with 2.8–4.2% based on *cytb* gene (511–513 base pair sequences). Because Arribas et al. (2018) used the lower base pair sequence of *cytb* gene and this part of *cytb* gene had higher variability, they found higher genetic distance than the present study. Besides, the genetic distance was found 1.8–2.8% between *D. parvula* and *D. adjarica*, 6.2–6.8% between *D. parvula* and *Darevskia* sp. and 5.4–6.8% between *D. adjarica* and unnamed *Darevskia* sp. for combined mitochondrial genome with 1048–1052 base pair in the present study. Tarkhnishvili (2012) stated that the genetic distance ranged from 2 to 15% for *cytb* gene with 1051 base pair in the *Darevskia* group. According to this result, the genetic distances was adequate that three mitochondrial group (*D. parvula*, *D. adjarica* and unnamed *Darevskia* sp.) indicated phylogenetic trees in the present study were separate species.

The nuclear gene variability was found lower, and the nuclear gene did not show any discrimination among populations of red-bellied lizard in the present study. Because of this, nuclear gene displayed discordance with mitochondrial gene. This discordance used to determine phylogenetic relationships were frequently encountered issue among animals within last decades (Funk and Omland 2003; Toews and Brelsford 2012). Our results indicated that mitochondrial genes displayed much more variability than the nuclear gene regions in the red-bellied lizard group. Variations in the mitochondrial DNA can be reduced more quickly than those in the nuclear loci during fast range expansions owing to its fourfold lower effective population size (Meiklejohn et al. 2007; Galtier et al. 2009), and because it may be affected regionally by selective sweeps (Ballard and Whitlock 2004). In addition, there are many different explanations; for instance, male-biased dispersal or disparities in range, size, or abundance between the hybridizing groups can promote the dispersal of mitochondrial DNA in the absence of a concordant movement of nuclear DNA (Funk and Omland 2003). This means that mitochondrial DNA will complete the process of lineage sorting, where ancestral polymorphisms are lost over time, faster than nuclear DNA, as this rate is inversely proportional to the effective population size (Funk and Omland 2003). Our results were congruent with this explanation. However, Arribas et al. (2018) found that there were two mutations between *D. parvula* and *D. adjarica* based on *mc1r* (melano-cortin 1 receptor) gene as against *rag1* gene used in the present study.

The species group formed at the end of phylogenetic analysis indicate geographical discrimination in the present study. *D. adjarica* haplogroups separated from *D. parvula* and unnamed *Darevskia* sp. haplogroups with Kaçkar and Karçal mountain ranges. *D. parvula* haplogroups separated from unnamed *Darevskia* sp. haplogroups with the Çoruh river arms passed on the Ardanuç and Şavşat detour (Figure 1). These geographic formations among species group showed isolation region, and they played important role in the mitochondrial separation of species groups. That is, our results showed that *D. adjarica* was distributed only coastal Black sea and near region to coast in the Eastern Black Sea. It was congruent with the results in the study of Darevsky and Eiselt (1980). However, Ilgaz (2009) found that Ardahan population was morphologically represented with *D. p. adjarica* subspecies. Accordingly, Arribas et al. (2018) concluded that Ardahan and Çermik (Şavşat) populations (inner side) represented with *D. adjarica*. However, this was not congruent with the geographical data in the present study. *D. parvula* haplogroups were represented with Ardanuç, Şavşat and east of centre Artvin province. Also, we found that Geçitli village represented with *D. parvula* haplogroup in the present study was to be distant about 10 km to Ardahan population indicated in the study of Arribas et al. (2018). However, they did not use the specimens of the Ardahan population in their phylogenetic analyses. Moreover, they used examples from less populations that could not solve both phylogenetic and geographic relations, and they used the specimens from inland only morphological discrimination. Our phylogeographic results showed that Hatila Valley, Salkımlı village, Ortaköy, Yusufeli, Horosan, Sarıkamış and Pazaryolu represented with unnamed *Darevskia* sp. The distribution area of the unnamed *Darevskia* sp. restricted between north of Aras river and south of Kaçkar mountains. According to morphlogical results in the study of Arribas et al. (2018), Hatila valley, Yusufeli, Şavşat populations were represented with *D. parvula*. However, some of these populations conflicted with unnamed *Darevskia* sp. in the present study. In fact, this situation indicates the necessity of more comprehensive comparative morphological and phylogenetic studies.

Ecological niche modelling can supply important information to solve taxonomic distinction and niche differentiation

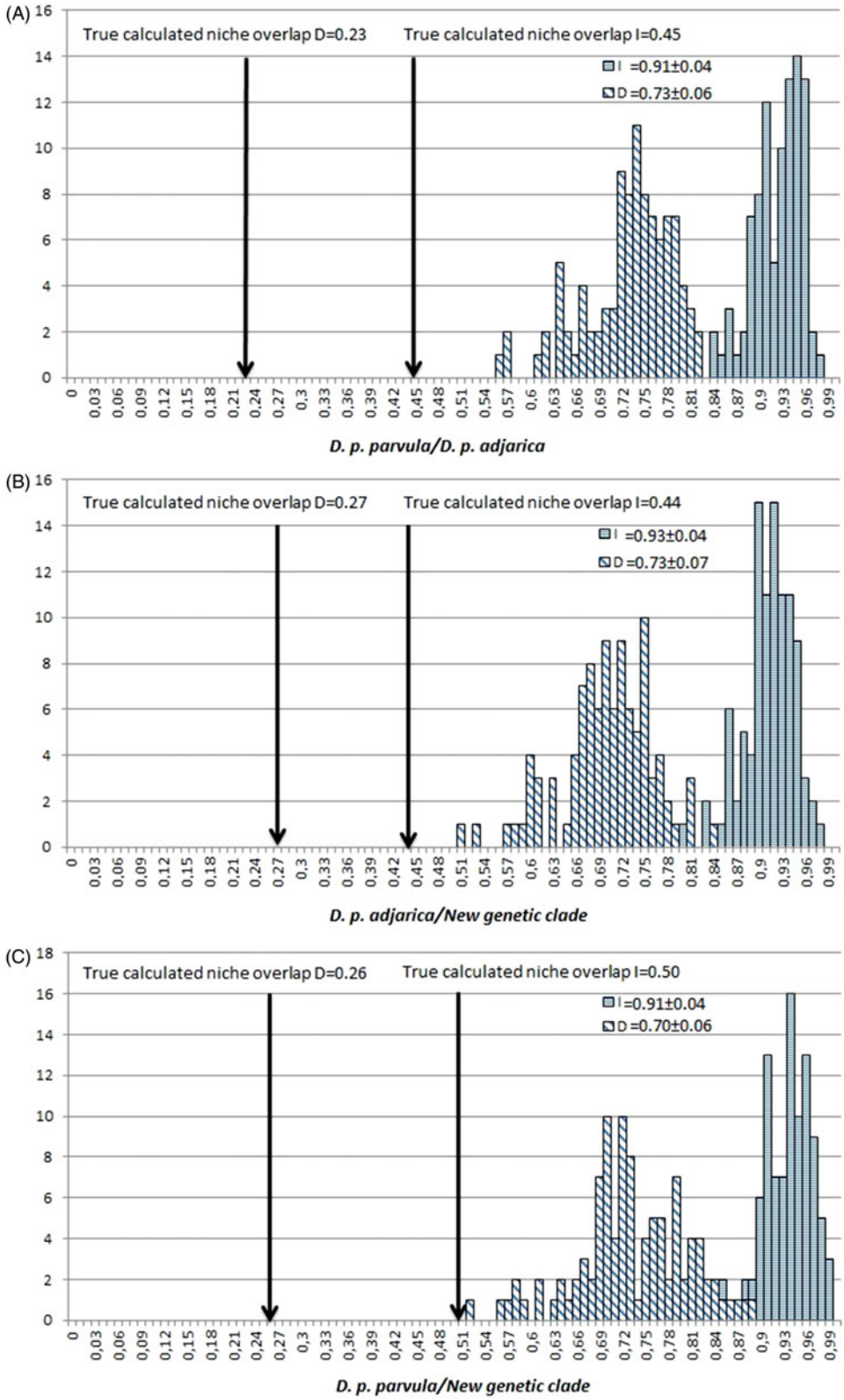

Figure 6. The results of the identity test performed using ENMTools. Black arrows refer to the true calculated niche overlap by ENMTools (D and I). The solid and hachured columns are calculated by replicates with identity test mode.

among close species group (Nakazato et al. 2010; Yousefkhani et al. 2016). Also, niche separation is very important for each species because each of them has different niche according to ecological knowledge. This situation shows that they need different requirements in their habitats. This study was performed for the first time that used ecological niche modelling to evaluate the ecological niche distinction among mitochondrial haplogroups of the red-bellied lizard in Turkey. According to the niche differentiation results, any overlap was not seen in terms of ecological niche and

geography among three mitochondrial haplogroups of red-bellied lizard. This is harmonious with the phylogenetic results of mitochondrial genome in the present study. Ecological niche differentiation, one of the factors for speciation, is confirmed by the niche identity test because the test supported the significant ecological niche divergence among mitochondrial haplogroups in the present study. The differentiation between *D. parvula* and *D. adjarica* in the species level were previously explained by mitochondrial DNA data (Arribas et al. 2018). However, they did not state that unnamed *Darevskia* sp. and ecological niche differentiation among species group appearing in their phylogenetic results.

In conclusion, our results indicate that mitochondrial DNA gene region discriminates the red-bellied lizard as three distinct species: *D. parvula*, *D. adjarica* and unnamed *Darevskia* sp. Also, ecological niche modelling results supported the mitochondrial phylogeny given in the present study. The geographical isolation zones among these three mitochondrial groups further strengthened this situation. The mitochondrial phylogeny results in the present study were congruent for *D. parvula* and *D. adjarica* given as species level in study of Arribas et al. (2018). Besides, phylogenetic, geographical and ecological niche findings in this study supported the study of Arribas et al. (2018) which said that *D. parvula* and *D. adjarica* were two distinct species. On the other hand, no *rag1* gene comparison of red-bellied lizard exists in the literature, and according to nuclear genes, red-bellied lizard exhibited no variations. *Darevkia* group lizards are very interesting for researchers in terms of the area they live in. This lizard group has a very large variety in a small area such as Caucasus. Congruent with this, because the distribution area of red-bellied lizard restricted with very small zone about 2.5 million hectares (Pontic zone). The lizard groups have high genetic diversity in this area of Turkey. Moreover, it is expected that the results of the current phylogeographic study will shed light on other studies which will be performed in this area in the future. Furthermore, other studies with red-bellied lizard groups will allow the nomenclature of the lizard group left unnamed in this study.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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