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THE ALLELIC VARIANTS IN MICROSATELLITE LOCI AND PHYLOGENETIC RELATIONSHIPS OF *Darevskia rudis* (BEDRIAGA, 1886) AND *D. bithynica* (MÉHELY, 1909) BASED ON MITOCHONDRIAL DNA IN TURKEY

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The spiny-tailed lizard, which has a series of taxonomic revisions, is one of the most common lizard species in Turkey. In this study, sequence data derived from three microsatellite loci (Du215, Du281, and Du323), two mitochondrial (16S rRNA and Cyt-*b*) genes and combined data were used to evaluate the taxonomic status of *Darevskia rudis* and *Darevskia bithynica* with new samples from all subspecies populations in Turkey. Our results indicated that the genetic variations of microsatellite loci were not specific to populations within species, and only minor differences separated *D. rudis* and *D. bithynica* populations. Furthermore, the markers we used for phylogenetic analyses (NJ, ML, MP, and BI) produced topologically similar trees based on 16S rRNA and Cyt-*b* while the combined data produced conflicting trees with the separate gene analyses. Finally, the basal relationships among the populations in Turkish populations *D. rudis* and *D. bithynica* were not resolved with this dataset, and we found a hard polytomy at the basis of the phylogeny.

Keywords: hard polytomy; Cyt-*b*; Anatolia; microsatellite; genetic diversity; 16S rRNA.

INTRODUCTION

Anatolia has many mountainous zones, which led to occurred many different habitats and climatic regions that have played an important role as geographical barriers for distribution of reptilians. Due to its geological status, Anatolia acted as either a bridge or barrier for the dispersal of animal species between Asia, Europe, and the region of Ethiopia (northeast Africa) through the Middle East (Tchernov, 1992).

The spiny-tailed lizard *Darevskia rudis*, has a wide distribution range in Anatolia and consists of six subspecies in its range. The nominal subspecies *D. rudis rudis* is distributed in northeastern Black Sea coastal region of Turkey. The second subspecies, *D. r. macromaculata*, is found between Şavşat town (in Artvin Province) and Ardahan Province in northeastern Anatolia. The third subspecies, *D. r. bischoffi*, is located in Rize and Artvin provinces in the Black Sea region of Turkey. The fourth one, *D. r. obscura*, is distributed from Kutul Plateau and between Geçitli Village and Bilbilan Plateau in Artvin Province. Recently, two other *D. rudis* subspecies were described by Arribas et al. (2013) *D. r. mirabilis* from Ovit Pass, Rize province in the Black Sea region and *D. r. bolkardaghica* from Ulukışla, Niğde province in the Central Anatolia region.

Darevskia bithynica is another species of *Darevskia*, which has a smaller distribution area than *D. rudis* in Anatolia. It was separated from *D. rudis* and raised to species rank, with two subspecies: *D. bithynica bithynica* and *D. b. tristis* by Arribas et al. (2013). The nominal subspecies *D. b. bithynica* lives in a small isolated area in Uludağ, Bursa northwestern Anatolia. The second subspecies of *D. bithynica*, *D. b. tristis*, is distributed in the western Black Sea region of Turkey.

In the literature, morphological (Bedriaga, 1886; Werner, 1902; Méhely, 1909; Lantz and Cyrén, 1936; Bodenheimer, 1944; Terentjev and Chernov, 1965; Darevsky, 1967; Başoglu and Baran, 1977; Böhme and Budak, 1977; Budak and Böhme, 1978; Böhme and Bischoff, 1984; Milto, 2010; Gabelaia et al., 2018), ecological (Gül et al., 2014) and osteological (Arribas et al., 2013) studies on *D. rudis* and *D. bithynica* have already been de-

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scribed. In addition, the latest nomenclature revision provides data on the current location of the type specimens of *Darevskia rudis* complex (Doronin, 2017). However, molecular data is limited, and the available studies with *D. rudis* and/or *D. bithynica* never included samples from all known subspecies (Ryabinina et al., 2003; Grechko et al., 2007, and Koç et al., 2017). Although Arribas et al. (2013) raised the level of the subspecies rank of *D. r. bithynica* to the species level; Koç et al. (2017) reported that the politomy of *D. rudis* complex continued. However, they did not sample *D. r. bolkardaghica* for partial sequences of mitochondrial DNA and they did not analyse neutral genetic markers, such as microsatellite loci. In the present study, we used partial sequences of both mitochondrial DNA (with more individuals) and microsatellite loci of all subspecies of *D. rudis* and *D. bithynica* so that with more comprehensive data we can better evaluate the phylogenetic relationships of these lizards in Turkey.

MATERIAL AND METHODS

Microsatellite DNA

Sampling. The samples from all subspecies populations distributed in Anatolia of the *D. rudis* complex were collected (based on the permission granted by the Turkey Ministry of Agriculture and Forestry, number of permission to capture: 72784983-488.04-70542). The identifications of the specimens were determined according to type specimens of *Darevskia rudis* complex (Doronin, 2017) (Fig. 1). Sampling locations are shown in Fig. 2A, and listed in Table 1. The lizards were caught by hand from August 2012 to July 2018. Tissue samples obtained from the third toes of lizards were stored in 96% ethanol for subsequent DNA analysis. The specimens are kept in the Zoology Research Lab of the Department of Biology, Karadeniz Technical University, Trabzon, Turkey.

The permits were obtained from the local ethics committee for treated in accordance with the guidelines (Karadeniz Technical University, 53488718-566/2015/38).

DNA extraction, PCR amplifications, and sequencing of microsatellite loci. The tissues belonged to 40 individuals from 24 localities were treated in 2 ml eppendorf tubes overnight at 56°C in 96% ethanol. DNA was extracted from clipped toes of the lizards using the Qiagen DNA tissue kit according the manufacturer's instructions.

Microsatellite primers were selected and modified as represented in the study of Korchagin et al. (2007) as following: Du215 (F: CAACTAGCAGTAGCTCTCCAGA and R: CCAGACAGGCCCCAACTT), Du281 (F: TTG CTAATCTGAATAACTG and R: TCCTGCTGAGAAA GACCA), Du323 (F: AAGCAGACTGTACAAAATCC CTA and R: ACTGATCTAAAGACAAGGTAAAAT). We also tried to amplify Du47 and Du418 loci for the lizards of *D. rudis* and *D. bithynica* populations. Despite a series of the PCR experiments, no results were obtained for Du47 locus. Du418 locus was not used in the present study because of contained degenerative GATA repeats. In the literature, there were insufficient data to obtain a successful result by studying the Du418 and Du47 loci for *Darevskia* genus. The same amplification conditions were used for all microsatellite loci (T_a = 48°C), except Du215 (T_a = 54°C). PCR amplifications were performed on DNA samples from all specimens. PCR amplifications were performed in total volumes of 40 µl with 20 µl 2X multiplex mix, 1 µl F primer, 1 µl R primer, 15 µl ddH_2O and 3 µl of genomic DNA as a template. Amplification of the microsatellite genes involved one cycle of 15 min at 95°C; 30 cycles of 20 sec at 95°C; 60 sec at the appropriate annealing temperature (48 – 54°C); and 2 min at 72°C; followed by one cycle of 10 min at 72°C (modified from Korchagin et al., 2007). 5 µl from each PCR was run on 8% polyacrylamide gel (to separate allelic variants for each locus) and run for 4 h at 80 V. A 50 bp ladder was used as a size marker. The amplification products were visualized by staining the DNA in the gel with ethidium bromide. Well-resolved individual PCR products, which corresponded to the two individual alleles of the locus were purified and sequenced by Macrogen Europe (Amsterdam, Netherlands).

Sequence analysis. We analyzed 160 – 226 bp region of the Du215, Du281, and Du323. All sequences of Du215, Du281, and Du323 loci were corrected and aligned. The unassembled sequences were screened for all possible sequence motifs of di-, tri-, and tetra- microsatellites with Primer3 (Faircloth, 2008). The microsatellites with tetra- repeat motifs were detected for Du215, Du281 and Du323 loci while it was also selected as di- for Du323. After the selection of microsatellites with motifs repeat, DNA sequences were aligned using CLC DNA Workbench 5 (CLC bio, Aarhus, Denmark). The haplotypes were submitted to GenBank for each locus (Table 2).

Mitochondrial DNA

Sampling. In total, 72 specimens were collected as the representative of both the *D. rudis* and *D. bithynica* distribution ranges in Turkey (shown in Fig. 2B, and listed in Table 1). Tissues for each of the individuals were preserved from the longest phalanges and stored in 96% ethanol at –20°C in the Zoology Research Lab at Karadeniz Technical University.

Fig. 1. The specimens of *D. rudis* complex: A, *D. b. bithynica*; B, *D. b. tristis*; C, *D. r. rudis*; D, *D. r. bolkardaghica*; E, *D. r. obscura*; F, *D. r. macromaculata*; G, *D. r. mirabilis*; H, *D. r. bischoffi*.

PCR amplifications and sequencing for mitochondrial DNA. For DNA extraction, each third toe sample was incubated in 2 ml eppendorf tubes overnight at 56°C. For the following steps, the Qiagen DNA tissue kit was used according the manufacturer's instructions. Two mitochondrial genes, 16S rRNA and Cytochrome-*b* (Cyt-*b*) were amplified using the 16SarL (Palumbi et al., 1991), 16SbrH (Palumbi et al., 1991) and L14724 and H15175 (Palumbi, 1996) primers, respectively. PCR conditions were performed on a total of 50 µl with 25 µl 2X multiplex mix, 1 µl F primer, 1 µl R primer, 22 µl ddH_2O and 1 µl of genomic DNA for both genes. PCR conditions for DNA amplification were: 3 min at 94°C for initial incubation; 35 cycles of 30 sec at 94°C; 30 sec at the appropriate annealing temperature (44 – 56°C); and 1 min at 72°C; followed by one cycle of 8 min at 72°C for 16S rRNA and it was 5 min at 94°C of initial incubation; 35 cycles of 60 sec at 94°C; 60 sec at the appropriate annealing temperature (50 – 55°C); and 1 min at 72°C; followed by one cycle of 70 sec at 72°C. Successfully amplified samples were sent for purification and sequencing to an external sequencing corporation (Macrogene, Netherlands).

Sequence alignment and phylogenetic analyses. The nucleotide sequences of each gene were aligned using the Bioedit (Thompson et al., 1997) software. Haplotypes were determined using TCS (Clement et al., 2000) program. The best-fitting models of both genes and com-

bined data selected by Akaike's Information Criterion (Akaike, 1974) were estimated with Modeltest 3.7 (Posada and Crandall, 1998). GTR was selected as the fit nucleotide substitution model in 16S rRNA and Cyt-*b* genes and HKY+G was selected as the fit nucleotide substitution model for combined data for phylogenetic analyses.

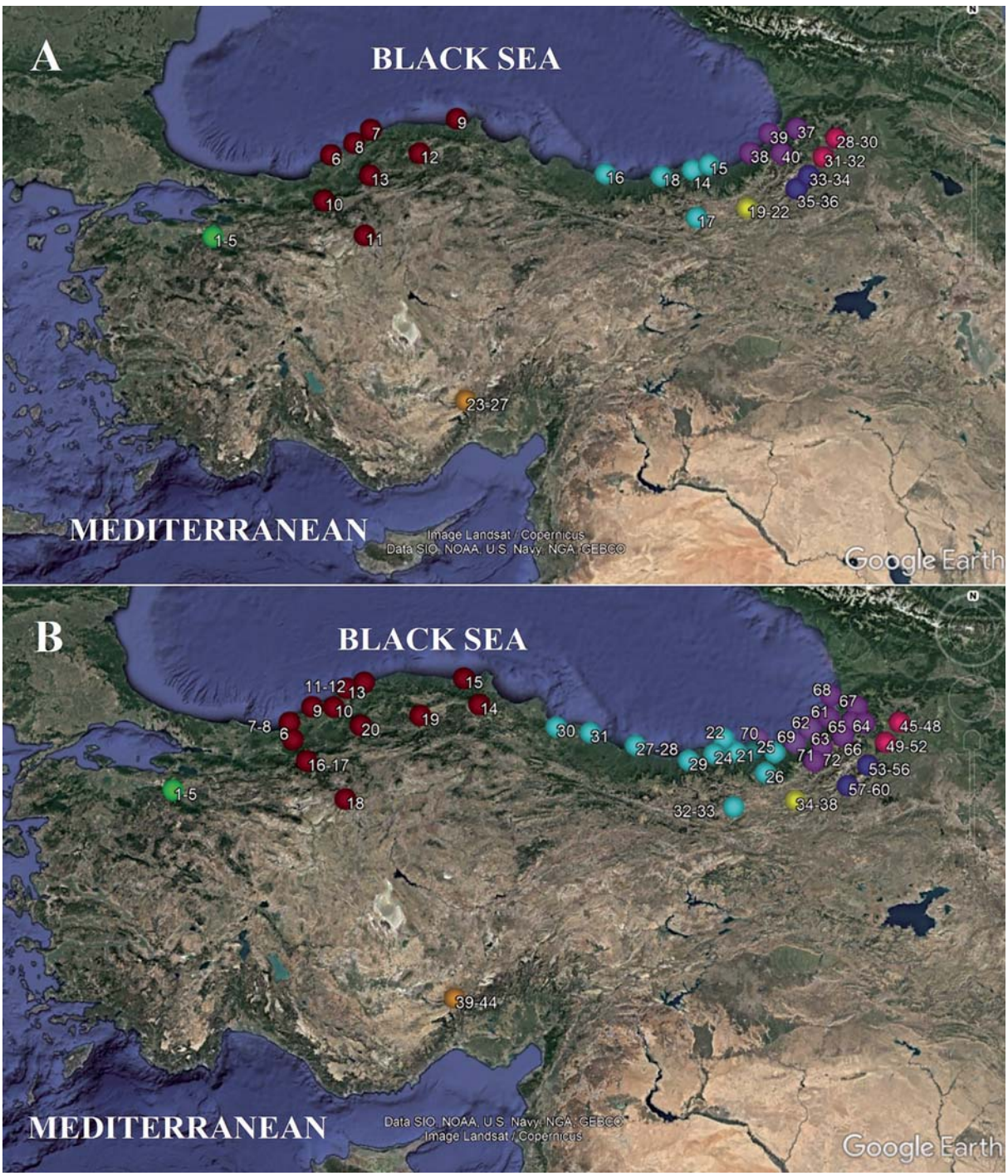

Fig. 2. Map shows the localities of the caught individuals based on the haplotypes of microsatellite DNA (A) and the haplotypes of mitochondrial DNA (B). The green circle represents *bithynica* while we use red for *tristis*, turquoise blue for *rudis*, yellow for *mirabilis*, orange for *bolkardaghica*, pink for *macromaculata*, purple for *obscura*, and violet for *bischoffi*. The detailed list of the localities are given in Table 1.

After confirming the suitability for combination of all of the sequences of the two genes, by performing the partition-homogeneity test (parsimony method by Farris et al. (1995) as implemented in PAUP*4.0b10 (Swofford, 2000)), we combined the data on these two genes (16S rRNA and Cyt-*b*). Phylogenetic analyses based on the two genes and combined data were performed by neighbour-joining (NJ), maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) methods. Firstly, PAUP (Swofford, 2000) was used for three phylogenetic analysis, neighbour-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML). Neighbour-joining (NJ) and Maximum Parsimony (MP) analyses were carried out using a heuristic search method (10,000 random addition replicates tree-bisection-reconnection, TBR, branch swapping) and bootstrap analyses for NJ and MP (Felsenstein, 1985) were applied. In NJ, MP, and ML analysis the support of the nodes was evaluated by bootstrapping with 1000 replicates. Secondly, MrBayes 3.2.3 (Ronquist and Huelsenbeck, 2003) was used for Bayesian inference (BI). In the BI analysis, the following settings were conducted: number of Markov Chain Monte Carlo (MCMC) generations = six millions for 16S rRNA and Cyt-*b*, and two millions for combined data, sampling frequency = 100. The first 25% trees were eliminated as the burn-in period. The majority consensus tree was computed from the remaining trees. The burn-in size was determined by checking convergence of -log likelihood (–ln *L*) using MrBayes 3.2.3 (Ronquist and Huelsenbeck, 2003). BI trees were determined based on the Bayesian posterior probability (BPP). In the BI analysis, we considered nodes with a BPP of 0.85 or greater as significant. In order to compare the genetic distances among specimens of *D. rudis* and *D. bithynica* we calculated the pairwise uncorrected *p*-distances for 16S rRNA and Cyt-*b* using MEGA v 6.0 (Tamura, 2013) (Table 3). *Phoenicolacerta laevis* (Gen-Bank accession number JN673190.1; Pavlicev et al., 2011, DQ461762.2; Pavlicev and Mayer, 2006) and *Darevskia parvula* (Gen-Bank accession number AF206195.1 (Fu, 1999) and U88609.3 (Fu et al., 2000) were used as the out groups for 16S rRNA and Cyt-*b*, respectively. The haplotypes were submitted to GenBank for each genes (Table 4).

TABLE 1. The Localities of the Specimens Used for Microsatellite and Mitochondrial DNA Analyses

Microsatellite			Mitochondrial DNA					
No.	Subspecies	Locality	No.	Subspecies	Locality	No.	Subspecies	Locality
1 – 5	*bithynica*	Bursa-Uludağ	1 – 5	*bithynica*	Bursa-Uludağ	34 – 38	*mirabilis*	Rize-Ovit Pass
6	*tristis*	Zonguldak-Kozlu	6	*tristis*	Düzce-Yığılca	39 – 44	*bolkardaghica*	Niğde-Ulukışla
7	*tristis*	Bartın-Kurucaşile	7 – 8	*tristis*	Zonguldak-Alaplı	45 – 48	*macromaculata*	Artvin-Çamgeçiti
8	*tristis*	Bartın-Amasra	9	*tristis*	Zonguldak-Kozlu	49 – 52	*macromaculata*	Artvin-Hocaköy
9	*tristis*	Sinop-Ayancık	10	*tristis*	Zonguldak-Çaycuma	53 – 56	*obscura*	Ardanuç-Bilbilan
10	*tristis*	Bolu-Center	11 – 12	*tristis*	Bartın-Kurucaşile	57 – 60	*obscura*	Ardanuç-Bilbilan (Lower part)
11	*tristis*	Bolu-Ankara	13	*tristis*	Bartın-Amasra	61	*bischoffi*	Artvin-Yeşilköy
12	*tristis*	Kastamonu	14	*tristis*	Sinop-Center	62	*bischoffi*	Artvin-Murgul
13	*tristis*	Karabük	15	*tristis*	Sinop-Ayancık	63	*bischoffi*	Artvin-Esenkıyı
14	*rudis*	Trabzon-Derecik	16 – 17	*tristis*	Bolu	64	*bischoffi*	Artvin-Hatila Vadisi
15	*rudis*	Trabzon-KTU	18	*tristis*	Bolu-Ankara	65	*bischoffi*	Artvin-Yanıklı
16	*rudis*	Ordu-Perşembe	19	*tristis*	Kastamonu	66	*bischoffi*	Artvin-Beşpare
17	*rudis*	Gümüşhane-Köse	20	*tristis*	Karabük	69	*bischoffi*	Rize-Ardeşen
18	*rudis*	Giresun-Görele	21	*rudis*	Trabzon-Çağlayan	70	*bischoffi*	Rize-İyidere
19 – 22	*mirabilis*	Rize-Ovit Pass	22	*rudis*	Trabzon-Yıldızlı	71	*bischoffi*	Rize-Ayder
23 – 27	*bolkardaghica*	Niğde-Ulukışla	23	*rudis*	Trabzon-Arsin	72	*bischoffi*	Rize-Fındıklı
28 – 30	*macromaculata*	Artvin-Çamgeçiti	24	*rudis*	Trabzon-KTU			
31 – 32	*macromaculata*	Artvin-Hocaköy	25	*rudis*	Trabzon-Sürmene			
33 – 34	*obscura*	Ardanuç-Bilbilan	26	*rudis*	Trabzon-Derecik			
35 – 36	*obscura*	Ardanuç-Bilbilan (Lower part)	27 – 28	*rudis*	Ordu-Perşembe			
37	*bischoffi*	Artvin-Lekoban	29	*rudis*	Giresun-Görele			
38	*bischoffi*	Rize-Ardeşen	30	*rudis*	Samsun-Merkez			
39	*bischoffi*	Artvin-Murgul	31	*rudis*	Samsun-Terme			
40	*bischoffi*	Artvin-Beşpare	32 – 33	*rudis*	Gümüşhane-Köse			

RESULTS

Microsatellite DNA

In total, 40 specimens of *D. rudis* and *D. bithynica* were analyzed using locus-specific PCR and DNA sequencing from Anatolia populations. Locus-specific PCR analysis showed that all individuals of *D. rudis* and *D. bithynica* were heterozygous for the loci, including Du215, Du281, and Du323 and contained several alleles that differed from each other regarding the length and structure of microsatellite clusters, and regarding single nucleotide variations in fixed positions of the flanking regions. All allelic variants of every locus divided into distinct groups according to the fixed nucleotide variations in the microsatellite flanking regions. In total 7, 8 and 8 genotypes differed for Du215 [Du215(rud)$_1$ – Du215(rud)$_7$], Du281 [Du281(rud)$_1$ – Du281(rud)$_8$] and Du323 [Du323(rud)$_1$ – Du323(rud)$_8$] in population frequencies were revealed, respectively (Table 2).

The sequences of Du215 locus were practically identical for all specimens in each population even though some differences were observed (Table 2). Du215 locus had 5 abundant and 2 rare-geographically restricted genotypes in *D. rudis* and *D. bithynica* populations. The third genotype, Du215(rud)$_3$, was the most common haplotype in our samples and it included GAT(GATA)$_7$GCAA repeats and it was shared by 11 individuals of *D. b. tristis* (ayan, amas and koz), *D. r. rudis* (per, ktu, gir, kos and der) and *D. r. bolkardaghica* (nig1, nig3 and nig4) populations. The (GATA)$_n$ repeats varied from 5 to 11 in most of *D. rudis* and *D. bithynica* populations while the nucleotide variations were observed only

TABLE 2. Allelic Variations of Microsatellite Containing Loci Du215, Du281, and Du323 in *D. rudis* and *D. bithynica* Species with the Corresponding Accession Numbers

Allelic variant	Size, bp	Structure of microsatellite cluster and names of subspecies and haplotypes	Nucleotide variations	Genbank Accession No.
Du215(rud)$_1$	226	GAT(GATA)$_{11}$GCAA *macromaculata* (cam1, cam2, and cam3), *obscura* (gec1, gec2, and gec3), *bischoffi* (lek, ard, mur, and bes)	C (–19)	MK496246
Du215(rud)$_2$	214	GAT(GATA)$_{11}$GCAA *tristis* (kur), *bithynica* (ulu2)	T (–19)	MK496247
Du215(rud)$_3$	221	GAT(GATA)$_7$GCAA *tristis* (ayan, amas, and koz), *rudis* (per, ktu, gir, kos, and der), *bolkardaghica* (nig1, 3 and 4)	C (–19)	MK496248
Du215(rud)$_4$	212	GAT(GATA)$_{10}$GCAA *tristis* (ank, kar, bolu, and kast), *mirabilis* (ovi3), *macromaculata* (hoc1), *obscura* (gec4)	C (–19)	MK496249
Du215(rud)$_5$	220	GAT(GATA)9GCAA *bolkardaghica* (nig2 and nig5), *macromaculata* (hoc2), *mirabilis* (ovi1, ovi2, and ovi4)	C (–19)	MK496250
Du215(rud)$_6$	213	GAT(GATA)$_5$GCAA *bithynica* (ulu1, ulu4, and ulu5)	C (–19)	MK496251
Du215(rud)$_7$	211	GAT(GATA)$_5$GCAA *bithynica* (ulu3)	T (–19)	MK496252
Du281(rud)$_1$	184	(GATA)$_5$(GAT)$_2$ *rudis* (der)	C (–13), C (–31)	MK496253
Du281(rud)$_2$	178	(GATA)$_5$(GAT)$_2$ *tristis* (ayan, kar, bolu, kast, kur, ank, koz, and amas), *rudis* (ktu and kose), *macromaculata* (cam2), *bolkardaghica* (nig3 and nig4)	G (–13), C (–31)	MK496254
Du281(rud)$_3$	185	(GATA)$_6$GAT *macromaculata* (hoc1 and hoc2), *obscura* (gec1, gec2, gec3, and gec4), *bolkardaghica* (nig1, nig2, and nig5), *mirabilis* (ovi1 and ovi4)	G (–13), C (–31)	MK496255
Du281(rud)$_4$	189	(GATA)$_7$(GAT)$_2$ *rudis* (gir)	G (–13), T (–31)	MK496256
Du281(rud)$_5$	181	(GATA)$_7$(GAT)$_2$ *rudis* (pers), *bithynica* (ulu3, ulu4, and ulu5)	G (–13), C (–31)	MK496257
Du281(rud)$_6$	178	(GATA)$_8$(GAT)$_2$ *bithynica* (ulu2)	G (–13), C (–31)	MK496258
Du281(rud)$_7$	181	(GATA)$_9$ *mirabilis* (ovi2 and ovi3)	G (–13), C (–31)	MK496259
Du281(rud)$_8$	190	(GATA)$_{10}$ *macromaculata* (cam1 and cam3)	G (–13), C (–31)	MK496260
Du323(rud)$_1$	191	(AC)$_7$...(GATA)$_1$3GATATAT(GA)$_4$ *bischoffi* (mur and ard), *macromaculata* (cam2 and cam3), *obscura* (gec1, gec2, gec3, and gec4)	A (–23), T (+39)	MK496261
Du323(rud)$_2$	186	(AC)$_7$...(GATA)$_1$3GATATAT(GA)$_4$ *macromaculata* (hoc2)	C (–23), T (+39)	MK496262
Du323(rud)$_3$	179	(AC)$_7$...(GATA)$_1$3GATATAT(GA)$_4$ *bischoffi* (bes and lek), *macromaculata* (hoc1 and cam1)	A (–23), C (+39)	MK496264
Du323(rud)$_4$	162	(AC)$_7$...(GATA)$_4$GAT(GATA)$_2$GATAGAT(GA)$_4$ *tristis* (koz, ank, amas, and kast), *rudis* (der, kos, per, and ktu), *bolkardaghica* (nig1, nig2, and nig3)	A (–23), T (+39)	MK496263
Du323(rud)$_5$	160	(AC)$_7$...(GATA)$_4$GAT(GATA)$_2$GATAGAT(GA)$_4$ *rudis* (gir)	C (–23), T (+39)	MK496265 MK496266
Du323(rud)$_6$	176	(AC)$_7$...(GATA)$_{10}$GAT(GA)$_4$ *bithynica* (ulu1, ulu2, ulu3, ulu4, and ulu5), *tristis* (bolu, kar, kur, and ayan), *bolkardaghica* (nig4 and nig5)	C (–23), T (+39)	
Du323(rud)$_7$	175	(AC)$_7$...(GATA)$_6$GAT(GATA)$_2$GATAGAT(GA)$_4$ *mirabilis* (ovi1, ovi2, and ovi3)	A (–23), T (+39)	MK496267
Du323(rud)$_8$	173	(AC)$_7$...(GATA)$_6$GAT(GATA)$_2$GATAGAT(GA)$_4$ *mirabilis* (ovi4)	C (–23), T (+39)	MK496268

in three individuals of *D. b. bithynica* and *D. b. tristis* in Du215(rud)2 and Du215(rud)$_7$ (Table 2).

The (GATA)$_n$ repeats in Du281 varied from 5 to 10 in most of *D. rudis* and *D. bithynica* populations. The third genotype, Du281(rud)$_3$, was the most common haplotype in our samples and it included (GATA)$_6$GAT repeats and shared by 15 individuals of *D. r. macromaculata* (hoc1 and hoc2), *D. r. obscura* (gec1, gec2, gec3, and gec4), *D. r. bolkardaghica* (nig1, nig2 and nig5), *D. r. mirabilis* (ovi1 and ovi4), and *D. r. bischoffi* (lek, mur, ard, and bes) populations. The nucleotide variations were observed only in two individuals of *D. r. rudis* in Du281(rud)$_1$ and Du281(rud)$_4$ lineages. Two nucleotide variations (G-C and C-T) were found in *D. r. rudis* populations from Trabzon province for Du281.

The (GATA)$_n$ repeats in Du323 varied from 4 to 13 in most of *D. rudis* and *D. bithynica* populations while (AC)$_n$ repeats remained constant. The genotypes of Du323(rud)$_5$ and Du323(rud)$_6$ the most common haplotypes in our samples and they include (AC)$_7$...(GATA)$_4$ GAT(GATA)$_2$GATAGAT(GA)$_4$ and (AC)$_7$...(GATA)$_{10}$ GAT(GA)$_4$ repeats, respectively and shared by 11 individuals in *D. rudis* and *D. bithynica* populations. The sequences of Du323 locus were separated into two groups according to a nucleotide in the flanking region (–23) while only *D. r. bischoffi* (bes and lek) and *D. r. macromaculata* (hoc1 and cam1) populations had a nucleotide difference C (+39) in Du323(rud)$_3$ lineage (Table 2).

Mitochondrial DNA

Phylogenetic relationships. The 16S rRNA and Cyt-*b* genes were successfully amplified in all *D. rudis* and *D. bithynica* populations. The 16S rRNA sequences were corrected and aligned, and a complete alignment (gaps were treated as missing data) with 561 bp length was obtained. Among the sequences of 72 individuals belonging to the *D. rudis* and *D. bithynica* populations, 23 distinct haplotypes were found and sequences for both strands were determined and sequence alignments were straight in 16S rRNA. All individuals from Uludağ and Ovit Pass had a 2 bp insertion, while all specimens from Kurucaşile, Sinop, and Niğde had a 1 bp insertion in the same position. The Cyt-*b* sequences were aligned and a complete alignment (gaps were treated as missing data) of 479 bp length was obtained. Among the 72 sequences

TABLE 3. Comparisons of Uncorrected *p*-Distances (in %) for 16S rRNA and Cyt-*b*

No.		1	2	3	4	5	6	7	8	9	10	11
						16S rRNA						
1	Lineage A1-1	—										
2	Lineage A1-2	0.3	—									
3	Subclade A2	0.7	0.4	—								
4	Subclade A3	0.5	0.2	0.2	—							
5	Clade B	0.7	0.4	0.4	0.2	—						
6	Subclade C1	1.0	0.9	0.9	0.7	0.5	—					
7	Subclade C2	1.1	0.8	0.4	0.6	0.4	0.5	—				
8	Subclade C3	1.3	0.9	0.9	0.7	0.5	0.6	0.5	—			
9	Clade D	0.7	0.4	0.4	0.2	0.0	0.5	0.4	0.5	—		
10	Clade E	0.7	0.4	0.4	0.2	0.0	0.5	0.4	0.5	0.0	—	
11	Subclade F1	1.1	0.8	0.8	0.6	0.4	0.9	0.8	0.7	0.4	0.4	—
12	Subclade F2	1.9	1.6	1.6	1.4	1.1	1.7	1.6	1.5	1.1	1.1	1.1
No.		1	2	3	4	5	6	7	8	9	10	
						Cyt-*b*						
1	Subclade A1	—										
2	Subclade A2	0.5		—								
3	Subclade A3	1.7		1.7	—							
4	Subclade B1	3.2		3.2	4.0	—						
5	Lineage B2-1	3.0		3.0	3.3	3.1	—					
6	Lineage B2-2	3.3		3.3	3.6	3.5	2.7	—				
7	Subclade C1	2.9		2.9	3.8	3.8	3.5	3.8	—			
8	Lineage C2-1	3.8		3.8	4.4	3.8	5.0	4.6	2.5	—		
9	Lineage C2-2	3.3	3.3	3.9	4.6	4.2	4.3	2.4	2.5	—		
10	Lineage C3-1	4.4	4.4	4.6	4.9	4.4	4.7	2.5	3.9	3.1	—	
11	Lineage C3-2	3.9	3.9	4.1	4.2	4.2	4.3	2.2	3.1	2.9	2.8	

of the *D. rudis* and *D. bithynica* populations, 33 distinct haplotypes were detected. In Cyt-*b*, there were no insertion and deletion. The phylogenetic analyses of 16S rRNA and Cyt-*b* genes using four different optimality criteria yielded slightly different topologies, and because of the tree topologies similarity only the BI tree is shown in Fig. 3 and 4.

The populations of *D. rudis* and *D. bithynica* formed 6 clades (Clades A – F) for 16S rRNA in Turkey, with unresolved basal relationships.

Clade A is divided into three subclades [Subclade A1 (*D. r. bischoffi*), Subclade A2 (*D. r. macromaculata*), and Subclade A3 (*D. r. obscura*)] (NJ, ML, and MP BS = 43, 63, and 57, respectively, and BPP = 0.9). Subclade A1 has two lineages (Lineages A1 and A2). Lineage A1 has two haplotypes (yes and yan3) of *D. r. bischoffi* from Artvin Province (NJ, ML, and MP BS = 38, 63, and 57, respectively, and BPP = 0.9). Subclades A2 and A3 has only one haplotype (hoca3 and cam1, respectively) of *D. r. macromaculata* from Artvin provinces (NJ, ML, and MP BS = 45, –, and –, respectively, and BPP = 0.9).

TABLE 4. List of the Haplotypes with Their Locality Names and the Corresponding Accession Numbers for the Mitochondrial DNA (16S rRNA and Cyt-*b*)

No.	Haplotype	Subspecies	Locality	Genbank Accession Number	
				16S rRNA	Cyt-*b*
1	ulu1	*bithynica*	Bursa-Uludağ-1	MK496223	MK503099
2	ulu2	*bithynica*	Bursa-Uludağ-2	MK496224	MK503100
3	ulu3	*bithynica*	Bursa-Uludağ-3	—	MK503101
4	yig	*tristis*	Düzce-Yığılca	MK496229	MK503106
5	ala1	*tristis*	Zonguldak-Alaplı-1	—	MK503107
6	ala2	*tristis*	Zonguldak-Alaplı-2	—	MK503108
7	koz	*tristis*	Zonguldak-Kozlu	—	MK503104
8	kur1	*tristis*	Bartın-Kurucaşile-1	MK496225	MK503109
9	kur2	*tristis*	Bartın-Kurucaşile-2	—	MK503110
10	amas1	*tristis*	Bartın-Amasra	MK496230	MK503102
11	bolu1	*tristis*	Bolu-Center-1	MK496226	—
12	bolu2	*tristis*	Bolu-Center-2	MK496227	—
13	sin1	*tristis*	Sinop-Center	MK496228	MK503111
14	sin2	*tristis*	Sinop-Ayancık	MK496232	MK503112
15	ank	*tristis*	Bolu-Ankara	—	MK503103
16	kast	*tristis*	Kastamonu	MK496231	MK503105
17	cag	*rudis*	Trabzon-Çağlayan	—	MK503120
18	yil	*rudis*	Trabzon-Yıldızlı	—	MK503121
19	ars	*rudis*	Trabzon-Arsin	MK496238	MK503122
20	der	*rudis*	Trabzon-Derecik	MK496239	MK503118
21	pers1	*rudis*	Ordu-Perşembe-1	MK496235	MK503115
22	pers2	*rudis*	Ordu-Perşembe-2	—	MK503116
23	gir	*rudis*	Giresun-Görele	MK496236	MK503117
24	sam	*rudis*	Samsun-Terme	MK496237	MK503119
25	ovi1	*mirabilis*	Rize-Ovit Pass-1	MK496240	MK503123
26	ovi5	*bolkardaghica*	Rize-Ovit Pass-5	—	MK503124
27	nig1	*bolkardaghica*	Niğde-Ulukışla-1	MK496233	MK503113
28	nig2	*bolkardaghica*	Niğde-Ulukışla-2	MK496234	—
29	nig3	*bolkardaghica*	Niğde-Ulukışla-3	—	MK503114
30	cam1	*macromaculata*	Artvin-Çamgeçiti-1	—	MK503127
31	cam2	*macromaculata*	Artvin-Çamgeçiti-2	—	MK503128
32	hoc3	*macromaculata*	Artvin-Hocaköy-3	MK496242	—
33	gec1	*obscura*	Ardanuç-Bilbilan Plateu-1	MK496241	MK503125
34	gec2	*obscura*	Ardanuç-Bilbilan Plateu-2	—	MK503126
35	yes	*bischoffi*	Artvin-Yeşilköy	MK496243	MK503130
36	yan	*bischoffi*	Artvin-Yanıklı	MK496244	MK503131
37	kem	*bischoffi*	Artvin-Kemalpaşa	MK496245	MK503129

Clade B has only one haplotype (ovi1) of *D. r. mirabilis* from Rize provinces (NJ, ML, and MP BS = 100, 100, and 100, respectively, and BPP = 1.0).

Clade C consisted of *D. r. rudis* populations and it has three subclades (Subclades C1, C2, and C3), (NJ, ML, and MP BS = 45, 55 and 52, respectively, and BPP = 0.9). Subclade C1 has two haplotypes (der and ars) of *D. r. rudis* from Trabzon (NJ, ML, and MP BS = 43, 55, and 56, respectively, and BPP = 0.9). Subclade C2 has only one haplotype (sam) of *D. r. rudis* from Samsun (NJ, ML, and MP BS = 44, –, and 52, respectively, and BPP = 0.9) while Subclade C3 consists of two haplotypes (per1 and gir) of *D. r. rudis* from Ordu and Giresun provinces (NJ, ML, and MP BS = 41, 54, and 54, respectively, and BPP = 0.9).

Clade D has only one haplotype (nig1) of *D. r. bolkardaghica* from Niğde province (NJ, ML, and MP BS = = 100, 100, and 52, respectively, and BPP = 1.0) and Clade E has the other haplotype (nig2) of *D. r. bolkardaghica* (NJ, ML, and MP BS = 100, 100, and 52, respectively, and BPP = 1.0).

Clade F consisted of two subclades (Subclades F1 and F2) (NJ, ML, and MP BS = 42, 75 and 52, respectively, and BPP = 0.8). Subclade F1 consisted of *D. b. tristis* populations and it has eight haplotypes (amas1, bolu1, bolu2, sin, yig, kur1, kast, and ayan) from Bartın, Bolu, Düzce, Kastamonu, and Sinop provinces (NJ, ML, and MP BS = 33, 76, and 52, respectively, and BPP = = 0.8) while Subclade F2 consisted of *D. b. bithynica* population and it has two haplotypes (ulu1 and ulu2) from Bursa province (NJ, ML, and MP BS = 33, 75, and 52, respectively, and BPP = 1.0).

The genetic distance values of the 16S rRNA are concordant with the tree topologies, and the values of *p*-distances among the Clades (A-F) were low (Table 3). The *p*-distance values were ranged from 0.0% [Clade D (nig1) and Clade E (nig2) which two haplotypes of *D. r. bolkardaghica*; Clade B (ovi1) and Clade D (nig1) and Clade B (ovi1) and Clade E (nig2)] to 1.9% [Lin-

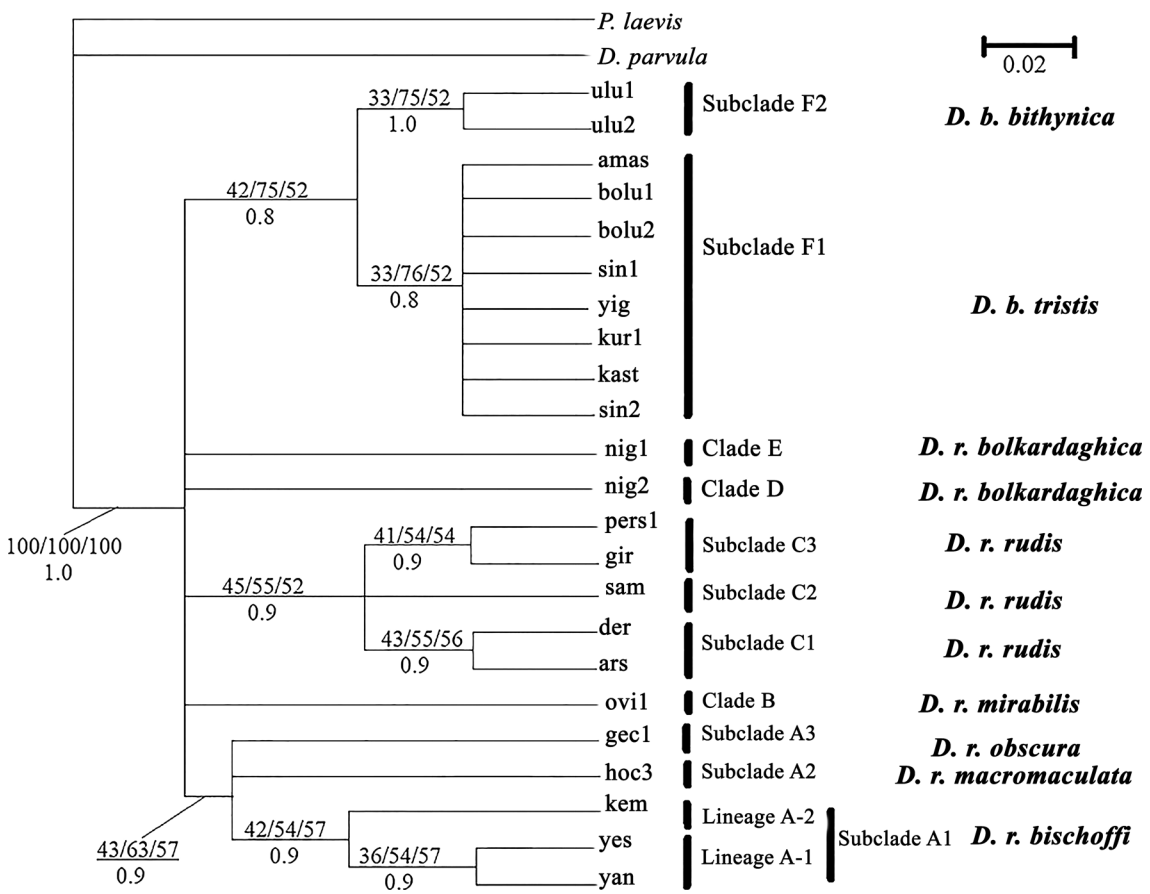

Fig. 3. Bayesian tree of a 561-bp sequence of 16S rRNA. Numbers above branches represent bootstrap support for NJ/ML/MP (1000 replicates) inherence, and numbers below branches indicate Bayesian Posterior Probabilities.

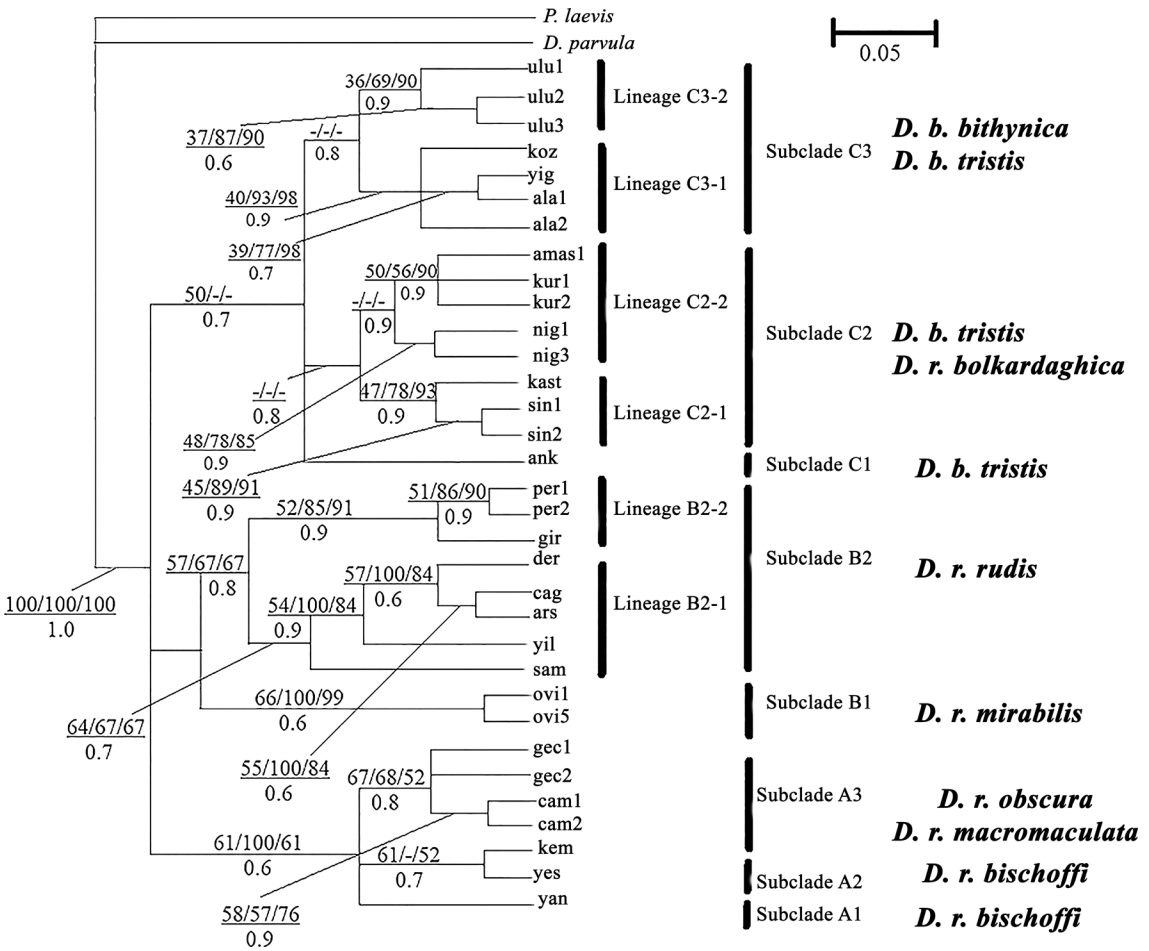

Fig. 4. Bayesian tree of a 479-bp sequence of Cyt-*b*. Numbers above branches represent bootstrap support for NJ/ML/MP (1000 replicates) inherence, and numbers below branches indicate Bayesian Posterior Probabilities.

eage A1 (yes and yan) and Subclade F2 (ulu1 and ulu2)] for *D. rudis* and *D. bithynica* populations in 16S rRNA.

Anatolian populations of *D. rudis* and *D. bithynica* formed 3 clades (Clades A, B, and C) for Cyt-*b* (Fig. 4). As in the 16S RNA, basal relationships are not resolved.

Clade A includes three subclades (Subclades A1, A2, and A3). Subclade A1 has only one haplotype (yan) of *D. r. bischoffi* from Artvin province. Subclade A2 consists of two haplotypes (kem and yes) of *D. r. bischoffi* from Artvin province while Subclade A3 consists of four haplotypes of *D. r. obscura* and *D. r. macromaculata*, respectively (gec1, gec2, cam1, and cam2), (NJ, ML, and MP BS = 61, 100, and 61, respectively, and BPP = 6.0).

Clade B has two subclades (Subclades B1 and B2). Subclade B1 has two haplotypes (ovi1 and ovi2) of *D. r. mirabilis* population from Ovit Pass, Rize province (NJ, ML, and MP BS = 66, 100, and 99, respectively, and BPP = 6.0).

Subclade B2 consisted of two lineages (Lineages B2-1 and B2-2) which was represented *D. r. rudis* populations. Lineage B2-1 includes five haplotypes (der, cag, ars, yil, and sam) of *D. r. rudis* from Trabzon and Samsun provinces while Lineage B2-2 includes three haplotypes (per1, per2, and gir) of *D. r. rudis* from Ordu and Giresun provinces (NJ, ML, and MP BS = 57, 67, and 67, respectively, and BPP = 8.0).

Clade C is divided three subclades (Subclades C1, C2, and C3), (NJ, ML, and MP BS = 50, –, and –, respectively, and BPP = 7.0). Subclade C1 has only one haplotype (ank) of *D. b. tristis* from Ankara province (NJ, ML, and MP BS = 100, 100, and 100, respectively, and BPP = = 7.0). Subclade C2 consists of two lineages (Lineages C2-1 and C2-2). Lineage C2-1 has three haplotypes (kast, sin1, and sin2) of *D. b. tristis* from Kastamonu and Sinop provinces (NJ, ML, and MP BS = 49, 78, and 93, respectively, and BPP = 8.0). Lineage C2-2 has five

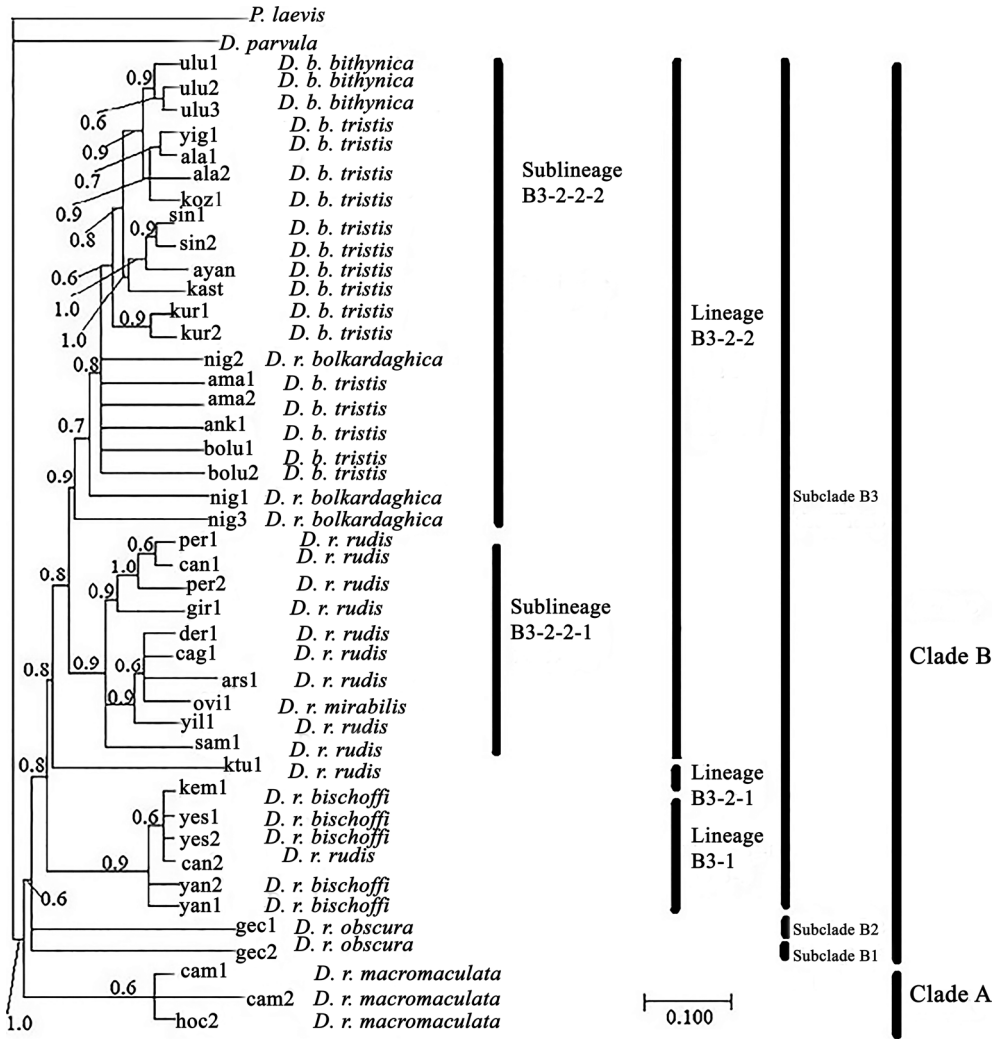

Fig. 5. Bayesian tree of a 865-bp sequence of combined data. The numbers on branches indicate Bayesian Posterior Probabilities.

haplotypes (amas1, kur1, kur2, nig1, and nig3) of *D. b. tristis* from Bartın and *D. r. bolkardaghica* from Niğde provinces (NJ, ML, and MP BS = 50, 56, and 90, respectively, and BPP = 8.0). Subclade C3 consists of two lineages (Lineages C3-1 and C3-2). Lineage C3-1 includes four haplotypes (koz, yig, ala1, and ala2) of *D. b. tristis* from Zonguldak and Düzce provinces while Lineage C3-2 includes three haplotypes (ulu1, ulu2, and ulu3) of *D. b. bithynica* from Bursa province (NJ, ML, and MP BS = 41, 69, and 90, respectively, and BPP = = 8.0).

The genetic distance values of the Cyt-*b* supported the tree topologies and the values of *p*-distances among the Clades A – F were low (Table 3). The *p*-distances were 3.4 between Clade A (*D. r. bischoffi*, *D. r. macromaculata*, and *D. r. obscura*) and Clade B (*D. r. mirabilis* and *D. r. rudis*) while it was 4.0 between Clades A and C (*D. r. bolkardaghica*, *D. b. tristis*, and *D. b. bithynica*). The *p*-distance was 4.3 between Clades B and C.

The phylogenetic analyses of combined data using four different optimality criteria yielded conflicted topologies, and only the BI tree is shown in Fig. 5. The populations of *D. rudis* and *D. bithynica* from Turkey formed 2 clades (Clades A and B) for combined data, with unresolved basal relationships.

Clade A consisted of *D. r. macromaculata* populations (cam1, cam2, and hoc2) while Clade B is divided into three subclades [Subclade B1 (*D. r. obscura*), Subclade B2 (*D. r. obscura*), and Subclade B3 (*D. b. bithynica*, *D. b. tristis*, *D. r. rudis*, *D. r. mirabilis*, *D. r. bolkardaghica*, and *D. r. bischoffi*)] (BPP = 0.9) based on combined data. Subclades B1 and B2 consisted of two haplo-

types (gec2 and gec1, respectively) of *D. r. obscura*. Subclade B3 has two lineages (Lineages B3-1 and B3-2). Lineage B3-1 has 5 haplotypes (kem1, yes1, yan1, yan2, and yus1) of *D. r. bischoffi* and a haplotype (can2) of *D. r. rudis*. Lineage B3-2 has two sublineages (Sublineages B3-2-1 and B3-2-2). Sublineage B3-2-1 has only one haplotype (ktu) of *D. r. rudis*. Sublineage B3-2-2 has two sublineages (Sublineage B3-2-2-1 and B3-2-2-2). Sublineage B3-2-2-1 has 9 haplotypes (per1, can1, per2, gir1, der1, cag1, ars1, yil, and sam1) of *D. r. rudis* and a haplotype (ovi1) of *D. r. mirabilis*. Sublineage B3-2-2-2 has 3 haplotypes (ulu1, ulu2, and ulu3) of *D. b. bithynica*, 15 haplotypes (yig1, ala1, ala2, koz1, sin1, sin, ayan, kast, kur1, kur2, amas1, amas2, ank1, bolu1, and bolu2) of *D. b. tristis* and 3 ones (nig1, nig2, and nig3) of *D. r. mirabilis*.

Both genes and combined data showed a hard polytomy in the interrelationships among *D. rudis* and *D. bithynica* populations, and that the divergence between them is not significant to separate them at the species level.

DISCUSSION

Microsatellite DNA

The remarkable taxon specificity of tandemly organized satellite repeats might reflect on the functioning and evolution of living organisms (Elder and Turner, 1995; Grechko et al., 2006). There is no study to reveal that the variances of Du215, Du281, and Du323 loci are specific to a particular bisexual population among the subspecies populations of *D. rudis* and *D. bithynica* although some parthenogenetic species of the genus *Darevskia* are studied with microsatellite loci (Korchagin et al., 2007; Malysheva et al., 2007; Vergun et al., 2014; Osipov et al., 2016; Girnyk et al., 2017). The mate among bisexual specimens of *D. rudis* and *D. bithynica* gave rise to high variable rate than some parthenogenetic species of *Darevskia* genus for Du215, Du281, and Du323 loci. This may be due to the fact that sexual reproduction increases the population level genetic diversity, when in comparison with parthenogenetic (and asexual) reproduction. Although the rate of expected genetic diversity was higher in bisexual species among the populations of *D. rudis* and *D. bithynica*, the genetic variations were not specific to populations, and genetic variations were included in all populations of *D. rudis* and *D. bithynica* with some differences.

Korchagin et al. (2007) reported that the two polymorphic loci (Du215 and Du281) including six allelic variants and three monomorphic loci (Du323, Du418, and Du47) for 65 individuals of *Darevskia* unisexualis; Malysheva et al. (2007) reported that a polymorphic locus, including at least three allelic variants (Du215) for 138 individuals of *Darevskia armeniaca*; Vergun et al. (2014) reported that three polymorphic locus (Du215, Du281 and Du323) including the alleles that varied from three to seven for 111 individuals of *Darevskia dahli* and Osipov et al. (2016) reported that three polymorphic loci (Du215, Du281 and Du323) including the alleles that the highest number of alleles was five for 42 individuals of *Darevskia rostombekovi*. On the other hand, *D. rudis* and *D. bithynica* (bisexual species) had 7, 8 and 8 allelic variants in Du215, Du281, and Du323 loci, in the present study. The bisexual species contained more allelic variants than parthenogenetic species brings to mind that parthenogenetic populations evolve more slowly than sexual forms, owing to a reduction or absence of recombination (Parker and Selandedr, 1976).

The allelic variants belonging to Du215, Du281, and Du323 [$Du215(rud)_1$ – $Du215(rud)_7$; $Du281(rud)_1$ – $Du281(rud)_8$; $Du323(rud)_1$ – $Du323(rud)_8$, respectively] had similar lengths and structures of microsatellite clusters in *rudis* and *bithynica* populations of Turkey (Table 2). Although some allelic variants exhibited variations in repeat numbers, the allelic variants of $Du215(rud)_3$, $Du281(rud)_2$, $Du281(rud)_5$, and $Du323(rud)_4$ were common in *rudis* and *bithynica* populations. Vieira et al. (2016) reported that repeat polymorphisms were usually caused by the addition or deletion of the entire repeat units or motifs. Although the repeat polymorphisms, different individuals may exhibit variations in repeat numbers (Vieira et al., 2016), *rudis* and *bithynica* populations have similar lengths and structures of microsatellite clusters in the present study.

The nucleotide variations in $Du215(rud)_2$ and $Du215(rud)_7$ were observed only in three individuals of *D. b. bithynica* and *D. b. tristis* populations. The populations of *bithynica* and *tristis* had variations apart from *rudis* populations and began separating for the Du215 locus. Although Malysheva et al. (2007) reported that Du215(arm) contained not only GATA but also GACA repeats, which were available in *D. dahli* and it was absent in *D. unisexualis*. Our results had no GACA repeats for Du215 locus for *D. rudis* and *D. bithynica* populations. This suggests that any *D. rudis* individual did not mate with individuals having GACA repeats in Du215 and the gene exchange of GATA continued among its populations. On the other hand, $Du323(rud)_3$ had a nucleotide difference in *rudis* (*D. r. bischoffi* and *D. r. macromaculata*) populations apart from *bithynica* populations. Additionally, Du323 locus was separated into two groups, which consisted both of *rudis* and *bithynica* populations, based on the nucleotide difference in (–23).

Apart from the Du215 and Du323 loci, the nucleotide variations were observed only in two individuals of *D. r. rudis* in Du281(rud)$_1$ and Du281(rud)$_4$. Two nucleotides were detected in *D. r. rudis* populations from Trabzon province for Du281.

Although genetic variations with repeat polymorphisms and the nucleotide differences in flanking regions were observed in all loci (Du215, Du281, and Du323) of *D. rudis* and *D. bithynica* populations, no difference was detected specific to all haplotypes of a population. It is well known that several bisexual lizard species freely hybridize in the Caucasus (Orlova, 1978; Darevsky, 1967) while the parthenogenic *Darevskia* species has a hybrid origin (Murphy et al., 2000; Ciobanu et al., 2002). There is free hybridization in bisexual lizard species and populations of *D. rudis* and *D. bithynica* share similar differences in flanking regions in the same loci, similar length, and structure of microsatellite clusters in our study.

In the present study, the evaluated sequences of microsatellite loci might be significant for understanding of genetic variability in the Spiny-tailed lizard populations apart from unisexual species. Intraspecific variations of Du215, Du281, and Du323 loci sequences were studied in *D. rudis* and *D. bithynica* populations for the first time. Our results pointed that the microsatellite sequences of *D. bithynica* were similar to *D. rudis* with some differences for Du215, Du281, and Du323 loci.

Mitochondrial DNA

D. rudis and *D. bithynica* clades were clustered into six groups based on the *p*-distances of 16S rRNA. Although *D. bithynica* has been raised to species level with two subspecies rank (*D. b. bithynica* and *D. b. tristis*) based on the external morphology and osteology (Arribas et al., 2013), the values of *p*-distances among the clades were low (Table 3). The *p*-distance values were ranged from 0.0% (*D. r. bolkardaghica* and *D. r. mirabilis*) to 1.9% (*D. r. bischoffi* and *D. b. bithynica*) for 16S rRNA. However, the *p*-distance was not sufficient to qualify it as a separate species.

The values of *p*-distance for Cyt-*b* among clades were relatively higher than the values of the genetic distance in 16S rRNA (Table 3). This high rate may be because the Cyt-*b* is a fast-evolving gene than 16S rRNA. The *p*-distances were 3.4 between *D. r. bischoffi*, *D. r. macromaculata* and *D. r. obscura* and *D. r. mirabilis* and *D. r. rudis* while it was 4.0 between *D. r. bolkardaghica*, *D. b. tristis* and *D. b. bithynica*. According to Kornilious et al. (2011), such conditions were a result of high gene flow rate. Although the combined data set had different topologies of phylogenetic trees, the *p*-distances of 16S rRNA and Cyt-*b* were low.

These two mitochondrial markers showed similar genealogies depending on the genetic distance values. Our results are in agreement with previous studies, which showed that the genetic distances of RAPD and new inter-MIR-PCR did not exceed 0.8% between *D. r. bischoffi* and *D. r. obscura* (Ryabinina et al., 2003). In addition, the genetic distances of *D. r. tristis*, *D. r. bischoffi* and *D. r. obscura* calculated were very low and the closer relationship was exposed among these subspecies (Grechko et al., 2007). Moreover, Mayer and Lutz (1989) investigated the albumins (LDH-2, MDH, MP-1, and PGM-1) with the immunological technique Micro-Complement-fixation for the lizards of *rudis* from Ordu (Yalıköy) and Gümüşhane (Zigana Pass) Provinces and *bithynica* from Bursa (Uludağ) and Kastamonu Provinces (Center). Mayer and Lutz (1989) reported that the structures of albumin were identical for *rudis* and *bithynica* lizards.

On the other hand, Gabelaia et al. (2018) reported that *D. r. obscura* from Georgia was morphologically as distinct from *D. r. rudis* as from the other nominal species based on threedi-mensional geometric morphometrics. Although Gabelaia et al. (2018) suggested that *D. r. obscura* was morphometrically distinct from *D. r. rudis*, we did not find a strong separation to qualify *D. r. obscura* as a separate species based on our microsatellite and mitochondrial DNA data in Turkey.

Koç et al. (2017) reported that the Uludağ population of *D. b. bithynica* and Ovit Pass population of *D. r. mirabilis* also seem to carry an island population structure based on their lower values of heterozygosity than the other populations. Besides, Böhme and Bischoff (1984) reported that if *bithynica* population (Uludağ) was different from *tristis* populations as noted in previous studies, *bithynica* had a structure as an island population. In the present study, we found that low *p*-distances based on 16S rRNA and Cyt-*b*, which shows that the gene exchanges continue among *rudis* and *bithynica* populations (Table 3, Fig. 5).

Tarkhnishvili et al. (2013) reported that in mountain regions, both spatial isolation and differential selection are potentially important factors of speciation. Although *D. b. bithynica* and *D. r. bolkardaghica* live in mountainous regions, the results of the present study show that they continue gene exchange with other subspecies. Compatible with the results of Koç et al. (2017), *D. b. bithynica* was begining to differentiate but these differentiations were not sufficient for speciation according to the findings of the present study. In addition, the gene exchange of the *D. r. mirabilis* populations is still ongoing with other populations.

Although multilocus phylogenetic studies are frequently preferred in the literature, elucidating the evolu-

tionary history of some relationships are difficult. Lack of resolution in a phylogenetic tree is usually represented as a polytomy, and adding more data (loci and taxa) resolves the phylogenetic tree in cases of soft polytomies. However, there are other cases of hard polytomies that can not be resolved with more data. Hard polytomy is often interpreted as a simultaneous adaptive radiation of lineages in the history of a clade. In cases of the hard polytomies, the phylogenetic trees include short internodes coupled with low nodal support, and conflicting topologies recovered by different methods, even with large and informative data sets (Olave et al., 2015). In parallel with these explanations, the markers we used for phylogenetic analyses (NJ, ML, MP, and BI) produced topologically similar trees based on 16S rRNA and Cyt-*b* while the combined data produced conflicting trees with the separate gene analyses.

In this study, the phylogenetic relationships of the populations, which contained all subspecies of *D. rudis* and *D. bithynica* showing the distribution in Turkey, were evaluated based on microsatellite and mitochondrial DNA markers for the first time. The basal relationships among the populations in Turkish populations *D. rudis* and *D. bithynica* were not resolved with this large and informative dataset, and we found a hard polytomy at the basis of the phylogeny. According to our results of microsatellite data, the phylogenetic analyses of 16S rRNA, Cyt-*b* and combined data, and the values of *p*-distance of 16S rRNA and Cyt-*b*, we temporarily suggest that *D. rudis* complex is still going on because the speciation process has not been completed. In addition, further taxonomical studies are needed for this species complex with the other subspecies *D. r. svanetica* from Georgia and Russia and *D. r. chechenica* from Azerbaijan, Georgia, and Russia.

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