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RESEARCH ARTICLE

Phylogenetic relationships of *D. rudis* (Bedriaga, 1886) and *D. bithynica* (Mehely, 1909) based on microsatellite and mitochondrial DNA in Turkey

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ABSTRACT

The spiny-tailed lizard of the genus *Darevskia* have a series of taxonomic revisions, but still their phylogenetic relationships remain uncertain. In the present study, we have assessed taxonomic relationships among *Darevskia bithynica* and *Darevskia rudis* populations through estimation of phylogenetic relationships among 96 specimens using microsatellite DNA (*Du215*, *Du281*, *Du323* and *Du418* loci) and 53 specimens using mtDNA (16S rRNA and cytb) from main populations in Turkey. Although *D. b. bithynica* and *D. r. mirabilis* were separated based on the PCoA analysis at low level from other *D. rudis* and *D. bithynica* populations, the distance values of Nei's genetic distance, Nei's unbiased genetic distance, Fst and Linear Fst were not high among taxa in microsatellite DNA. On the other hand, our phylogenetic analyses (NJ, ML, MP and BI) did not separate *D. rudis* and *D. bithynica* populations. Finally, most of the topologically identical trees of phylogenetic analyses and microsatellite results showed that the extant populations of *D. rudis* and *D. bithynica* were found to be polytomy. Based on our molecular phylogenetic study, *D. rudis* complex is still ongoing revisions.

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Introduction

Darevskia rudis and *Darevskia bithynica* have broad distribution areas in Turkey. *Darevskia rudis* is represented with 6 subspecies in Turkey. *Darevskia rudis rudis* (Bedriaga 1886) occurs in the northeastern Black Sea coastal region of Turkey. *Darevskia rudis bischoffi* (Böhme & Budak 1977) populations are located in Rize and Artvin provinces in northeastern Anatolia, while *Darevskia rudis obscura* (Lantz & Cyren 1936) populations are from Kutul Plateau and between Geçitli Village and Bilbilan Plateau in the Artvin province. The populations of *D. rudis macromaculata* (Darevsky 1967) are presented between Şavşat town (in Artvin province) and Ardahan province. Arribas et al. (2013) reported the new subspecies, *Darevskia rudis mirabilis* and *Darevskia rudis bolkardaghica* from Ovit Pass in northeastern Anatolia and Karagöl, Ulukïşla, Niğde province, respectively. Other species, *D. bithynica*, has two subspecies. *Darevskia bithynica bithynica* (Mehely 1909) lives in a small isolated area in Uludağ, Bursa northwestern Anatolia, while *Darevskia bithynica tristis* (Lantz & Cyrén 1936) occurs in the western Black Sea region of Turkey.

Formerly, many morphological studies were carried out to determine the taxonomic status of both species inhabiting Turkey (Lantz & Cyrén 1936; Terent'ev & Chernov 1965; Bodenheimer 1944; Darevsky 1967; Böhme & Budak 1977; Başoğlu & Baran 1977; Budak & Böhme 1978; Böhme & Bischoff 1984; Bedriaga 1886; Werner 1902; Boulenger 1904; Méhely 1909). In addition to morphological characteristics, ecologic, osteological and molecular data were used in recent years. Ryabinina et al. (2003) revealed the low genetic differences between *D. r. bischoffi* and *D. r. obscura*. In addition, Grechko et al. (2007) investigated the closer relationship of *D. r. tristis, D. r. bischoffi* and *D. r. obscura* based on their genetic distances. On the other hand, Milto (2010) investigated the morphology and ecology of *D. r. tristis* and reported the possible closer relationship between *D. r. tristis* and *D. raddei*. Finally, Arribas et al. (2013) reviewed the systematics of *D. rudis* based on the external morphology and osteology of the specimens from the main populations in Turkey. In their study, *D. r. bithynica* is raised to species rank, with *Darevskia bithynica bithynica* and *Darevskia bithynica tristis* subspecies. In addition, *D. r. mirabilis* from Ovit Pass, Trabzon and *D. r. bolkardaghica* from Karagöl, Ulukïşla, Niğde, were described as two new subspecies of *D. rudis* by Arribas et al. (2013). Although their morphological, ecological and osteological comparisons were comprehensive, they did not explain the phylogenetic relationships of these lizards. However, molecular markers are advocated to identify the genetic relationships of populations and to choose the most objective criteria for the development of taxon systematics on the basis of their phylogeny (Hillis 1987).

The purpose of the present study is to appraise the phylogenetic relationships of the *D. bithynica* and *D. rudis* samples from the great main distribution areas of Anatolia by the results of microsatellite and mitochondrial DNA for the first time. The phylogenetic analyses of the microsatellite and

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Supplemental data for this article can be accessed here.

mitochondrial DNA structures serves to test the results or hypothesis that were performed in the previous studies with comprehensive sampling materials.

Material and methods

Collection of the samples

A total of 96 specimens were collected from different localities in Turkey (Table 1). The specimens are kept in the Zoology Lab of the Department of Biology, KTU, Trabzon, Turkey. The animals were treated in accordance with the guidelines of the local ethics committee (KTU.53488718-566/2015/38).

DNA extraction, PCR amplifications and reading peaks for microsatellite DNA

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 extracted using the Qiagen DNA isolation kit following manufacturer's instructions. The microsatellite primers were used (Table 2) as described in the study of Korchagin et al. (2007). While three of microsatellite loci primers (*Du215*, *Du281* and *Du418*) were optimized under the same reaction conditions, one of them (*Du215*) was optimized under different reaction conditions. PCR amplifications were carried out using DNA samples from 96 individuals (Table 1). PCRs were performed in total volumes of 20 µL with 10 µl 2× multiplex mix, 0.5 µl F primer, 0.5 µl R primer, 7.5 µl ddH_2O and 1.5 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 s at 95 °C, 60 s 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).

Table 1. List of the samples used for determining of divergences of the microsatellite loci.

No	Subspecies	Localities	No	Subspecies	Localities
1	*D. b. bithynica*	Bursa-Uludağ-1	49	*D. r. mirabilis*	Rize-Ovit Pass -4
2	*D. b. bithynica*	Bursa-Uludağ-2	50	*D. r. mirabilis*	Rize-Ovit Pass -5
3	*D. b. bithynica*	Bursa-Uludağ-3	51	*D.r.macromaculata*	Artvin-Çamgeçiti-1
4	*D. b. bithynica*	Bursa-Uludağ-4	52	*D.r.macromaculata*	Artvin-Çamgeçiti-2
5	*D. b. bithynica*	Bursa-Uludağ-5	53	*D.r.macromaculata*	Artvin-Çamgeçiti-3
6	*D. b. tristis*	Düzce-Yığılca	54	*D.r.macromaculata*	Artvin-Çamgeçiti-4
7	*D. b. tristis*	Zonguldak-Alaplı-1	55	*D.r.macromaculata*	Artvin-Hocaköy-1
8	*D. b. tristis*	Zonguldak-Alaplı-2	56	*D.r.macromaculata*	Artvin-Hocaköy-2
9	*D. b. tristis*	Bartın-Kurucaşile	57	*D.r.macromaculata*	Artvin-Hocaköy-3
10	*D. b. tristis*	Bartın-Kurucaşile	58	*D.r.macromaculata*	Artvin-Hocaköy-4
11	*D. b. tristis*	Sinop-1	59	*D. r. obscura*	Ardanuç-Bilbilan Plateu-1
12	*D. b. tristis*	Sinop-2	60	*D. r. obscura*	Ardanuç-Bilbilan Plateu-2
13	*D. b. tristis*	Sinop-3	61	*D. r. obscura*	Ardanuç-Bilbilan Plateu-3
14	*D. b. tristis*	Sinop-4	62	*D. r. obscura*	Ardanuç-Bilbilan Plateu-4
15	*D. b. tristis*	Sinop-5	63	*D. r. obscura*	Ardanuç-Bilbilan Plateu lower part-1
16	*D. r. rudis*	Gümüşhane-Köse-1	64	*D. r. obscura*	Ardanuç-Bilbilan Plateu lower part-2
17	*D. r. rudis*	Gümüşhane-Köse-2	65	*D. r. obscura*	Ardanuç-Bilbilan Plateu lower part-3
18	*D. r. rudis*	Gümüşhane-Köse-3	66	*D. r. bischoffi*	Artvin-Çiftekemerköprü
19	*D. r. rudis*	Ordu-Ünye	67	*D. r. bischoffi*	Rize-İyidere-2
20	*D. r. rudis*	Ordu-Perşembe-1	68	*D. r. bischoffi*	Rize-İyidere-3
21	*D. r. rudis*	Ordu-Perşembe-2	69	*D. r. bischoffi*	Rize-Ayder
22	*D. r. rudis*	Giresun-Dereli	70	*D. r. bischoffi*	Rize-Fındıklı
23	*D. r. rudis*	Giresun-Görele	71	*D. r. bischoffi*	Rize-Ardeşen
24	*D. r. rudis*	Samsun-1	72	*D. r. bischoffi*	Artvin-Camili
25	*D. r. rudis*	Samsun-2	73	*D. r. bischoffi*	Artvin-Çifteköprü-1
26	*D. r. rudis*	Trabzon-Arsin	74	*D. r. bischoffi*	Artvin Hatila Valley-1
27	*D. r. rudis*	Trabzon-Center	75	*D. r. bischoffi*	Artvin-Esenkiyi-1
28	*D. r. rudis*	Trabzon-Arsin	76	*D. r. bischoffi*	Artvin-Esenkiyi-2
29	*D. r. rudis*	Samsun-Terme	77	*D. r. bischoffi*	Artvin-Kemalpaşa
30	*D. r. rudis*	Samsun-Canikli	78	*D. r. bischoffi*	Artvin-Murgul-1
31	*D. r. rudis*	Samsun Center-1	79	*D. r. bischoffi*	Artvin-Murgul-2
32	*D. r. rudis*	Samsun Center-2	80	*D. r. bischoffi*	Artvin-Y. Balaban-17
33	*D. r. rudis*	Trabzon-Düzköy	81	*D. r. bischoffi*	Artvin-Ortacalar-2
34	*D. r. rudis*	Trabzon-Çağlayan	82	*D. r. bischoffi*	Artvin
35	*D. r. rudis*	Trabzon-Yıldızlı	83	*D. r. bischoffi*	Artvin-Hatila Valley
36	*D. r. rudis*	Trabzon-Dernekpazarı	84	*D. r. bischoffi*	Artvin-Yanıklı -1
37	*D. r. rudis*	Trabzon-KTU	85	*D. r. bischoffi*	Artvin-Yanıklı -2
38	*D. r. rudis*	Trabzon-Sürmene	86	*D. r. bischoffi*	Artvin-Beşpare-1
39	*D. r. rudis*	Trabzon-Zigana	87	*D. r. bischoffi*	Artvin-Beşpare-2
40	*D. r. rudis*	Trabzon-Of	88	*D. r. bischoffi*	Artvin-Lekoban-1
41	*D. r. rudis*	Samsun-Bafra	89	*D. r. bischoffi*	Artvin-Lekoban-2
42	*D. r. rudis*	Gümüşhane	90	*D. r. bischoffi*	Artvin-Cancir-1
43	*D. r. rudis*	Trabzon-Derecik	91	*D. r. bischoffi*	Artvin-Cancir-2
44	*D. r. rudis*	Trabzon-Akçaabat	92	*D. r. bischoffi*	Artvin-Madenköy
45	*D. r. rudis*	Trabzon-Yomra	93	*D. r. bischoffi*	Artvin-Barhal-2
46	*D. r. mirabilis*	Rize-Ovit Pass-1	94	*D. r. bischoffi*	Ardeşen-Çamlıhemşin
47	*D. r. mirabilis*	Rize-Ovit Pass -2	95	*D. r. bischoffi*	Artvin-Yusufeli
48	*D. r. mirabilis*	Rize-Ovit Pass -3	96	*D. r. bischoffi*	Artvin-Yeşilköy

Table 2. Characterization of *Du215*, *Du281*, *Du323* and *Du418* loci isolated from *D. r. rudis*, *D. b. bithynica* and *D. r. mirabilis*.

			D.b. bithynica		*D. r. rudis*		*D. r. mirabilis*	
Locus	Primer Sequence (5′-3′)	Repeated Motif	He	Ho	He	Ho	He	Ho
Du215	F: CAACTAGCAGTAGCTCTCCAGA R: CCAGACAGGCCCCAACTT	$GAT(GATA)_9$	0.820	0.800	0.941	0.567	0.656	0.250
Du281	F: TTGCTAATCTGAATAACTG R: TCCTGCTGAGAAAGACCA	$GATA)_{10}TA(GATA)$	0.740	0.200	0.936	0.724	0.740	0.600
Du323	F: AAGCAGACTGTACAAAATCCCTA R: ACTGATCTAAAGACAAGGTAAAAT	$(GATA)_7GAT(GATA)_2$	0.719	0.250	0.931	0.655	0.840	1.000
Du418	F: AATGCAACAGGTGGATAATACTT R: TCTCTAATACAGCTTGCCATAAAT	$(GATA)_2GAT(GATA)_4GAT(GATA)_2$	0.640	0	0.943	0.414	0.750	0

F: "forward"; R : "reverse" primers; He : expected heterozygosities; Ho : observed heterozygosities.

After the amplification, the products were mixed with 1 µl loading dye (6×) and 5 µl 100-bp size markers onto a standard 1% agarose gel and run at 100 V. The amplification products were visualized by gel staining with ethidium bromide solution. Forward primers of the most successful products were provided as marked with different Colored fluorescent to determine DNA sizes in the next amplification. Each primer was diluted 10% with distilled water and they were avoided from light at −20 °C.

The next stage included the repeated amplifications under the same conditions, but forward fluorescent primers (e. g. FAM, VIC, NED and PED). These products were visualized by gel staining with the ethidium bromide solution. The most successful products were diluted to 2% with distilled water. Finally, the diluted products were purified with solution including formaldehyde, size standard and ddH_2O.

The sizes of the allelic variants of the PCR products were determined using the 3500 Genetic Analyzer with 600 LIZ size standard and they were compared with GeneMarker (Oakwood, USA). We calculated the number of alleles (N_A), observed (H_o), expected (H_e) heterozygosity for each locus and population with the GenAlEx v6.5 (Peakall & Smouse 2012) program.

All loci were evaluated using frequency-based population genetic analysis and spatial genetic analysis for *D. bithynica* and *D. rudis* species. The former includes allele frequency, heterozygosity, *F*-statistics (Fst and Linear Fst), Nei Genetic Distance (Nei genetic distance and unbiased Nei genetic distance) and Hardy–Weinberg equilibrium. The latter includes Principal Coordinates Ananlysis (PCoA) (Peakall & Smouse 2012).

Mitochondrial DNA

PCR amplifications for mitochondrial DNA

A 540 base pair fragment of the 16S rRNA gene and a 451 base pair fragment of the cytb gene for 53 specimens (Supplementary Table 1) were amplified using 16SarL and 16SbrH (Palumbi et al. 1991); L14724 and H15175 (Palumbi 1996) primers, respectively. Each 16S rRNA gene amplification involved an initial incubation 3 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at the appropriate annealing temperature (48–54 °C), and 1 min at 72 °C, followed by one cycle of 8 min at 72 °C. PCR amplifications for 16S rRNA were conducted as described by Guo et al. (2011). Each cytb gene amplification involved an initial incubation 5 min at 94 °C, 35 cycles of 60 s at 94 °C, 60 s at the appropriate annealing temperature (50–55 °C), and 1 min at 72 °C, followed by one cycle of 70s at 72 °C. PCR amplifications for cytb were conducted as described by Poulakakis et al. (2003). Amplified DNA segments were purified and sequenced by Macrogen Europe (Amsterdam, Netherlands).

Sequence alignment and phylogenetic analyzes

The nucleotide sequences of each gene were aligned using the Bioedit (Thompson et al. 1997) program. Haplotypes were determined for each gene using the TCS (Clement et al. 2000) program. GenBank accession numbers for each haplotype sequence are given in Supplementary Table 1. After confirming the suitability for the combination of all the sequences of two genes, by performing the partition-homogeneity test (the parsimony method by Farris et al. (1995) as implemented in PAUP [Swofford 2000]), we combined the data on these two genes for ML and BI. Phylogenetic analyses were based on the two genes (16S rRNA and cytb) separately and combined data. We conducted multiple complementary methods of data analysis, such as neighbour-joining (NJ), maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) phylogenetic approaches using PAUP (Swofford 2000) for NJ and MP, TREEFINDER (Jobb 2011) for ML and MrBayes 3.2.3 (Ronquist and Huelsenbeck 2003) for BI. NJ and 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. Transitions and transversions were equally weighted, and gaps were treated as missing data. The robustness of the resultant ML trees were evaluated using bootstrap analysis with 1000 replications. In the BI analysis, the following settings were conducted: number of Markov Chain Monte Carlo (MCMC) generations = six millions; sampling frequency =100; burn-in =25%. The burn-in size was determined by checking the convergence of –log likelihood (−ln L) using MrBayes 3.2.3 (Ronquist & Huelsenbeck 2003). NJ and MP trees were evaluated using bootstrap analyses with 1000 replicates and statistical support of the resultant BI trees was determined based on the Bayesian posterior probability (BPP). Best fit nucleotide substitution model was determined for each gene region with TREEFINDER (Jobb 2011) for ML and Modeltest 3.8 software (Posada & Crandall 1998) for NJ, MP 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 & Hillis 1993), and those between 50 and 70% as tendencies.

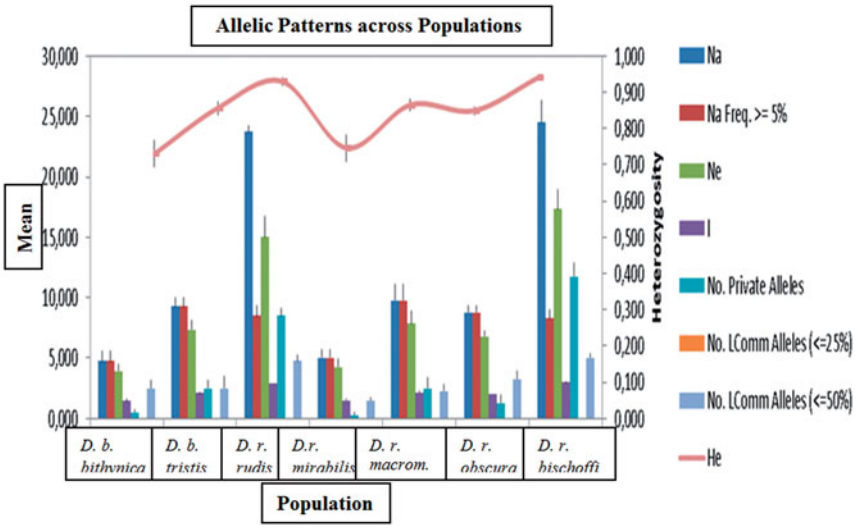

Figure 1. Allelic patterns across populations. Na: Number of different alleles; Na (Freq ≥5%): Number of different alleles with a frequency ≥5%; Ne: Number of effective alleles; I: Shannon's Information Index; No. Private Alleles: Number of alleles unique to a single population; No. LComm Alleles (≤25%): Number of Locally Common Alleles (frequency ≥5%) found in 25% or fewer populations; No. LComm Alleles (≤50%): Number of Locally Common Alleles (frequency ≥5%) found in 50% or fewer populations; He: expected heterozygosity.

In the BI analysis, we considered nodes with a BPP of 95% or greater as significant (Leaché & Reeder 2002). Uncorrected pairwise sequence divergences for each gene were calculated using MEGA 6.0 v (Tamura et al. 2013). *Phoenicolacerta laevis* (Gen-Bank accession number JN673190.1; Pavlicev et al. 2011 and 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 selected as the outgroups for 16S rRNA and cytb, respectively.

Results

Microsatellite DNA

The present study showed that all loci were polymorphic. The expected and observed values were determined for each taxon. The values of expected heterozygote were higher than the observed heterozygote for all loci (Table 2). Comparison of the highest expected and observed heterozygote values showed that *Du323* had the highest values while *Du418* had the lowest ones among the populations. *Darevskia b. bithynica* had 10 alleles for *Du215*, *Du281* and *Du418*, and 8 alleles for Du323 while *D. b.tristis* had 16 alleles for *Du215* and 20 for *Du281*, *Du418* and *Du323*. *Darevskia r. rudis* had 60 alleles for *Du215* and 58 for *Du281*, *Du323* and *Du418*. *Darevskia r. mirabilis* had 8 alleles for *Du215* and *Du418* and 10 for *Du281* and *Du323*. *Darevskia r. macromaculata* had 16 alleles for each locus, while *D. r. obscura* had 14 for each locus. *Darevskia r. bischoffi* had 60 alleles for *Du215*, *Du 281* and *Du418* and 50 for *Du323*. *Darevskia r. rudis* and *D. r. bischoffi* were not in the Hardy–Weinberg equilibrium for all loci, while *D. b. tristis* was also not in the Hardy–Weinberg equilibrium for *Du281*, *Du323* and *Du418* loci. Other taxa were in the Hardy–Weinberg equilibrium for each locus.

A number of different alleles with ≥5% frequency, number of effective alleles, a number of other different alleles and a number of locally common alleles ≤25% and ≤50% were calculated. Each population had the same size for common alleles for each locus. Apart from the common alleles, some populations had special sizes of alleles for each locus (Figure 1). According to the special sizes of alleles, *D. b. bithynica* and *D. r. mirabilis* had low differences of microsatellite DNA sizes, while *D. r. rudis* and *D. r. bischoffi* populations had high differences. The list based on the sizes of variant allele and their frequencies for *Du215*, *Du281*, *Du323* and *Du418* loci are given in Supplementary Tables 2 and 3. Genetic divergences among the populations were examined using GenAlEx v6.5 (Peakall & Smouse 2012) with Nei's genetic distance, Nei's unbiased genetic distance (Hedrick 2000), Fst (Hartl & Clark 1997) and Linear Fst (Slatkin 1995) parameters to reveal the genetic differences at different hierarchical levels. The main relationships were as follows:

1. According to the values of Nei's genetic distance, Nei's unbiased genetic distance, Fst and Linear Fst parameters, all populations were close to each other.
2. According to the Nei's genetic distance, Nei's unbiased genetic distance, Fst and linear Fst samples of *D. b. tristis* were close to *D. rudis* than *D. b. bithynica*.
3. Although *D. b. bithynica* and *D. r. mirabilis* seem to be distinguished from other populations (with not highly striking values) for PCoA analysis (Figure 2) using values of Fst and linear Fst (Table 3), they were not diverged from other populations by Nei's genetic distance, Nei's unbiased genetic distance.
4. Although *D. r. macromaculata* and *D. r. obscura* seem to be separated from other *D. rudis* populations for PCoA analysis using the values of Nei's genetic distance and Nei's unbiased genetic distance, they were not diverged from other populations by Fst and linear Fst.

Mitochondrial DNA

Phylogenetic analyses – sequence variation

A total of 517 homologous base pairs of the 16S rRNA sequences and 417 homologous base pairs of the cytb sequences for

Table 3. Data on the significant differences between the populations according to values of Nei's genetic distance, Nei's confidential genetic distance, Fst and Linear Fst. n1 and n2 represent number of the samples in ech population.

Population 1	Population 2	n1	n2	The values of Nei's genetic distance	The values of Nei's unbiased genetic distance	The values of Fst	The values of Linear Fst
D. b. bithynica	*D. b. tristis*	5	10	1.433*	1.033*	0.048*	0.051*
	D. r. rudis	5	30	1.508	1.183	0.051	0.054
	D. r. mirabilis	5	5	1.643	1.217	0.054	0.057
	D. r. macromaculata	5	8	2.002	1.529	0.067	0.072
	D. r. obscura	5	7	3.628	3.150	0.089	0.098
	D. r. bischoffi	5	31	1.473	1.112	0.047	0.050
D. b. tristis	*D. r. rudis*	10	30	0.534*	0.196*	0.004*	0.004*
	D. r. mirabilis	10	5	1.021	0.583	0.008	0.008
	D. r. macromaculata	10	8	1.162	0.677	0.023	0.024
	D. r. obscura	10	7	1.112	0.621	0.021	0.021
	D. r. bischoffi	10	31	0.988	0.615	0.025	0.026
D. r. rudis	*D. r. mirabilis*	30	5	1.169	0.805	0.029	0.030
	D. r. macromaculata	30	8	1.004	0.593	0.015	0.016
	D. r. obscura	30	7	1.050	0.633	0.017	0.018
	D. r. bischoffi	30	31	0.709	0.410	0.012	0.012
D. r. mirabilis	*D. r. macromaculata*	5	8	1.554	1.043	0.040	0.042
	D. r. obscura	5	7	1.294	0.777	0.034	0.035
	D. r. bischoffi	5	31	1.237	0.838	0.031	0.032
D. r. macromaculata	*D. r. obscura*	8	7	1.111	0.547	0.021	0.021
	D. r. bischoffi	8	31	1.072	0.626	0.020	0.020
D. r. obscura	*D. r. bischoffi*	7	31	1.067	0.615	0.021	0.021

*The values show that the populations of *D. b. tristis* and *D. r. rudis* closer than the populations of *D. b. bithynica* and *D. b. tristis*.

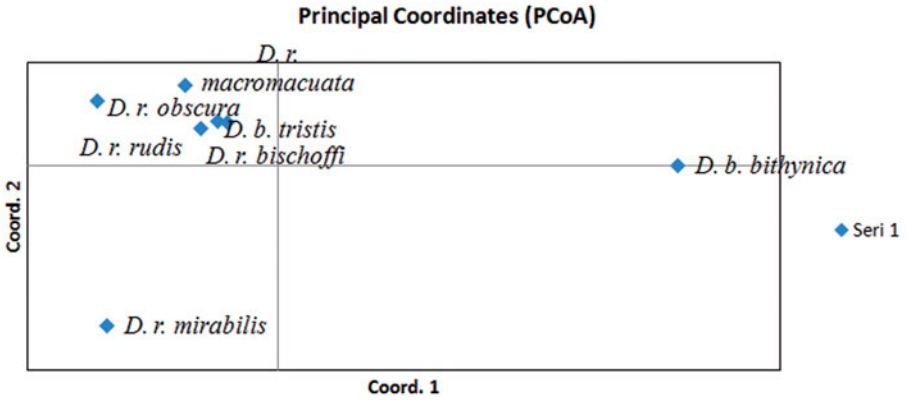

Figure 2. Results of Principal Coordinates Analysis (PCoA) according to values of Fst and Linear Fst.

53 individuals were obtained. Sequences for both strands were determined and sequence alignment was straight – all individuals from Uludağ and Ovit Pass possessed a 2 bp insertion in 16S rRNA gene, while all specimens from Kurucaşile and Sinop had a 1 bp insertion in the same gene. There were no insertion and deletion in cytb gene. In total, 12 mitochondrial haplotypes for 16S rRNA gene were identified and 19 haplotypes for cytb gene were recognized. When we combined both the 16S rRNA and cytb sequences, we identified 19 haplotypes. In the ML, the best fit model was selected by TREEFINDER (Jobb 2011) J2 (Jones et al. 1992) with a gamma-shape parameter for 16S rRNA and J2 for 1st, 2nd and 3rd codon positions of cytb with a gamma-shape parameter estimated cytb. Because of the best fit model similarity, the sequences of 16S rRNA and cytb were combined and GTR + G (Tavaré 1986; Nei & Kumar 2000) model was selected for combined sequences. In the NJ and MP analyses, the best fit model was selected by ModelTest 3.8, GTR + G (Tavaré 1986; Nei & Kumar 2000) for 16S rRNA and JC + G (Jukes and Cantor 1969; Nei & Kumar 2000) for cytb. Because of the differences of the best fit models, the sequences of 16S rRNA and cytb were not combined for NJ and MP. The parsimony analyses indicated the number of informative characters to be 27, with 53 variable characters being parsimony-uninformative for 16S rRNA. The number of the same characters to be 48 and 61 for cytb and 88 and 140 for combined data, respectively. In BI, the likelihood settings from the best-fit model were selected as JC (Jukes & Cantor 1969) in ModelTest 3.8 for 16S rRNA and cytb. Because of the best fit model similarity, the sequences of 16S rRNA and cytb were combined. JC + G (Jukes & Cantor 1969; Nei & Kumar 2000) model was selected for the combined data.

Phylogenetic relationships

NJ, MP, ML and BI phylogenetic analyses of each of the studied genes and the combined dataset gave very similar results and they showed only minor differences, mainly concerning relationships between these groups and their support values. The phylogenetic trees of the BI analysis of the 16S rRNA, cytb and combined data are shown Figure 3–5.

The phylogenetic analyses of 16S rRNA gene employing four different optimality criteria yielded very slightly different

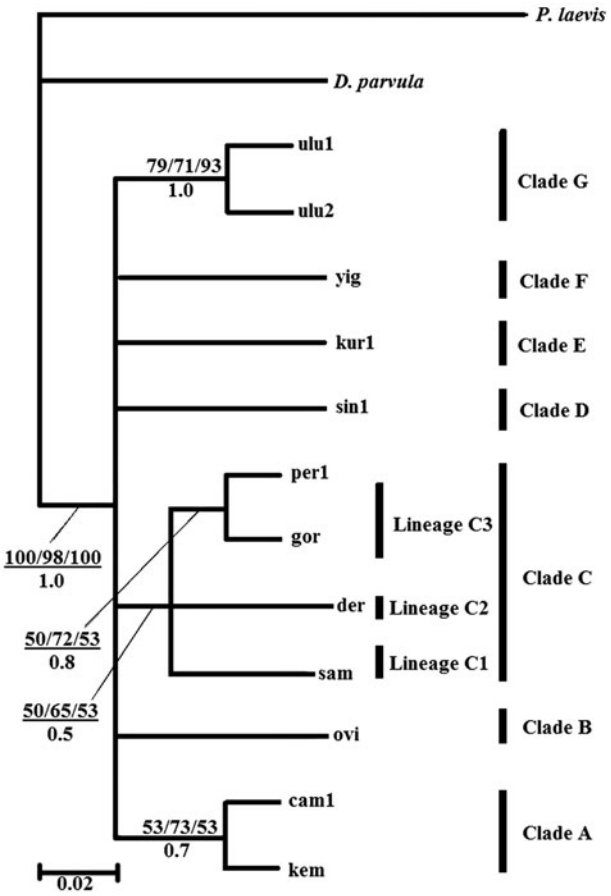

Figure 3. Bayesian tree of a 540-bp sequence of 16S rRNA for *D. bithynica* and *D. rudis*. Numbers above branches represent bootstrap support for NJ/ML/MP (1000 replicates) inherence, and numbers below branches indicate Bayesian Posterior Probabilities.

topologies, and only the BI tree is shown in Figure 3. Anatolian populations of *D. rudis* and *D. bithynica* formed 7 clades (Clades A-G) for 16S rRNA.

The main relationships were as follows for 16S rRNA. Clade A consists of two haplotypes (cam 1 and kem): samples between Şavşat town (in Artvin province) and Ardahan province and Kemalpaşa from Rize and Artvin provinces in northeastern Anatolia (NJ, ML and MP BS= 53, 73 and 53, respectively and BPP= 0.7). Clade B consists of ovi1 haplotype from Ovit Pass. Clade C was divided into three lineages (Lineages C1–C3) samples from Central and Eastern Black Sea (NJ, ML and MP BS= 50, 65 and 53, respectively and BPP= 0.5). Relationships among the samples of these lineages were unresolved. Lineage C1 includes sam haplotype. Lineage C2 includes der haplotype and Lineage C3 includes per1 and gor haplotypes (NJ, ML and MP BS= 50, 72 and 53, respectively and BPP= 0.8). Clades D, E and F consist of samples from the western Black Sea (yig, kur1 and sin1 haplotypes, respectively). Relationships among the samples of these clades were unresolved. Clade G consists of two haplotypes (ulu1 and ulu2) from Uludağ-Bursa (NJ, ML and MP BS= 79, 71 and 93, respectively and BPP= 1.00). The interrelationships among these groups are rather ambiguous, showing a polytomy for 16S rRNA.

As shown in Figures 4 and 5, Anatolian populations of *D. rudis* and *D. bithynica* form 2 clades (Clade A and Clade B) for cytb (NJ, ML and MP BS= –, 48 and –, respectively and BPP= 0.5) and combined data (ML BS= 99 and BPP= 1.0). The topologies of cytb with BS values in ML and combined data with BPP values in BI were well supported by the NJ, ML and MP with bootstrap values for cytb.

The main relationships were as follows for cytb and combined data:

1. Clade A includes two haplotypes (der and cag) from Trabzon (NJ, ML and MP BS= 87, – and 82, respectively) and BPP= 0.5 for cytb and (ML BS= 50 and BPP= 0.8 for combined data).
2. Clade B consists of *D. b. bithynica*, *D. b. tristis*, *D. r. mirabilis*, *D. r. macromaculata*, *D. r. obscura*, *D. r. bischoffi* and *D. r. rudis*. Clade B was divided into three subclades (NJ, ML and MP BS= –, 99 and –, respectively, and BPP= 0.7 for cytb and ML BS= 99 and BPP= 0.9 for combined data); the first one is Subclade B1 (sam) (NJ, ML and MP BS= 86, 99 and 98, respectively, and BPP= 1.0 for cytb and ML BS= 99 and BPP= 1.0 for combined data), second one is Subclade B2 (per1, can, gor, kos2) (NJ, ML and MP BS= 85, 74 and 65, respectively and BPP= 0.9 for cytb and ML BS= 89 and BPP= 1.0 for combined data) and third one is Subclade B3 (ulu1, ulu2, yig, ala1, ala2, kur1, sin1, ovi1, cam1, ard2, ard3 and kem) (NJ, ML and MP BS= –, 80 and –, respectively and BPP= 0.7 for cytb and ML BS= 88 and BPP= 0.9 for combined data).
3. Subclade B1 consists of a haplotype (sam) from Samsun Province (NJ, ML and MP BS= 86, 99 and 98, respectively and BPP= 1.0 for cytb and ML BS= 99 and BPP= 1.0 for combined data).
4. Subclade B2 was divided into two lineages (Lineage B2-1 and B2-2) (NJ, ML and MP BS= 85, 74 and 65, respectively and BPP= 0.9 for cytb and ML BS= 89 and BPP= 1.0 for combined data). Lineage B2-1 consists of two haplotypes (gor and kos2) from Giresun and Gümüşhane Provinces, respectively (NJ, ML and MP BS= 96, 76 and 81, respectively and BPP= 0.9 for cytb and ML BS= 89 and BPP= 1.0 for combined data). Lineage B2-2 consists of two haplotypes (per1 and can) from Trabzon (NJ, ML and MP BS= 98, 88 and 93, respectively, and BPP= 0.9 for cytb and (ML BS= 96 and BPP= 1.0 for combined data).
5. Subclade B3 was divided into six lineages (Lineage B3-1–B3-6) (NJ, ML and MP BS= –, 80 and –, respectively, and BPP= 0.7 for cytb and ML BS= 88 and BPP= 0.9 for combined data). Lineage B3-1 consists of a haplotype (kem) Kemalpaşa from Rize and Artvin provinces in northeastern Anatolia (NJ, ML and MP BS= 94, 70 and 71, respectively, and BPP= 0.9 for cytb and ML BS= 81 and BPP= 1.0 for combined data) and Lineage B3-2 consists of three haplotypes (cam1, ard2 and ard3) between Şavşat town (in Artvin province) and Ardahan province and Kutul Plateau and between Geçitli Village and Bilbilan Plateau in Artvin province (NJ, ML and MP BS= 72, 88 and 56, respectively, and BPP= 0.9 for cytb and ML BS= 63 and BPP= 0.9 for combined data), respectively. Lineage B3-3 consists of a haplotype (ovi1) from Ovit Pass (NJ, ML and MP BS= 86, 80 and 98, respectively, and BPP= 0.8 for cytb and ML BS= 90 and BPP= 1.0 for combined data); Lineage B3-4 consists of

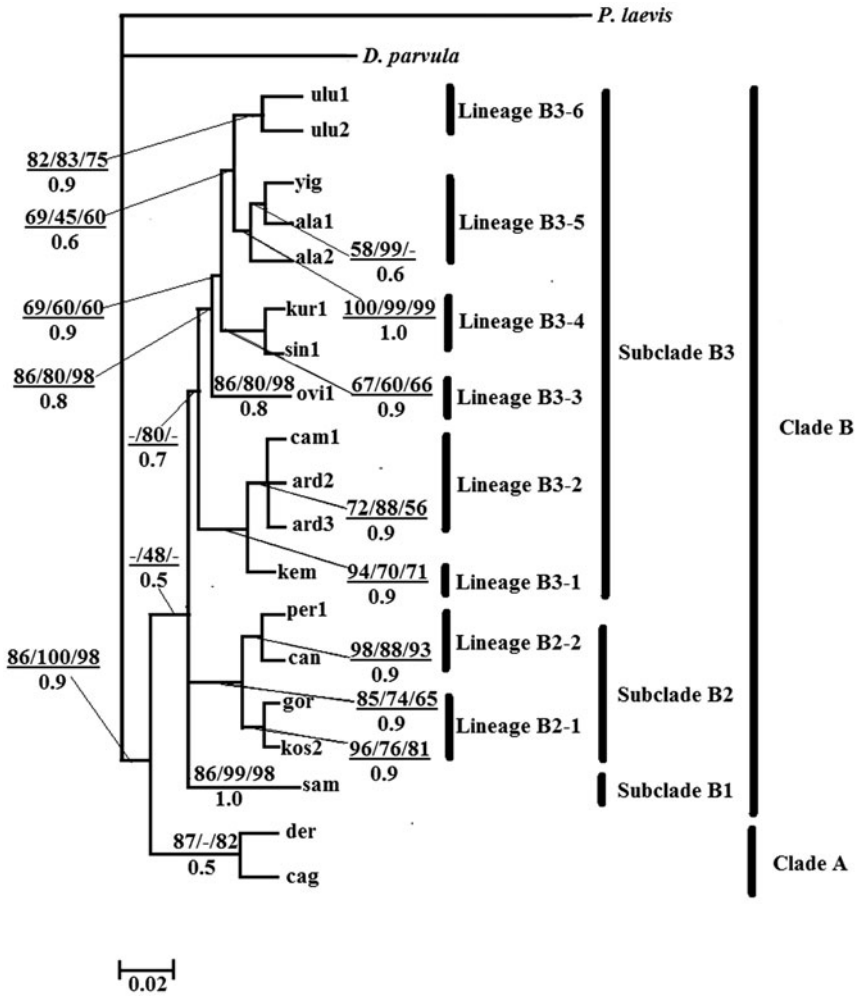

Figure 4. Bayesian tree of a 451-bp sequence of cytb for *D. bithynica* and *D. rudis*. Numbers above branches represent bootstrap support for NJ/ML/MP (1000 replicates) inherence, and numbers below branches indicate Bayesian Posterior Probabilities.

two haplotypes (kur1 and sin1) from Western Black Sea (NJ, ML and MP BS = 67, 60 and 66, respectively, and BPP = 0.9 for cytb and ML BS = 59 and BPP = 0.9 for combined data); Lineage B3-5 consists of three haplotypes (yig, ala1 and ala2) (NJ, ML and MP BS = 100, 99 and 99, respectively, and BPP = 1.0 for cytb and ML BS = 98 and BPP = 1.0 for combined data); Lineage B3-6 consists of two haplotypes (ulu1 and ulu2) from Uludağ-Bursa (NJ, ML and MP BS = 82, 83 and 75, respectively, and BPP = 0.9 for cytb and ML BS = 99 and BPP = 1.0 for combined data).

The interrelationships among these groups are rather ambiguous, showing a polytomy for cytb and combined data. All four phylogenetic analyses (NJ, ML, MP and BI) resulted in a polytomy where bootstrap and posterior probabilities were low.

Discussion

Microsatellite DNA

In the present study, differences in genetic structure among the populations of *D. bithynica* and *D. rudis* species were evaluated, and it was found that the number of microsatellite DNA size was very low in *D. b. bithynica* (8, 10) from Uludağ population and *D. r. mirabilis* (8, 10) from Ovit Pass population, while it was very high in *D. b. tristis* (16, 20) and the other *D. rudis* (14, 16, 50, 58, 60) populations.

In parallel with the low number of different microsatellite DNA sizes, the values of heterozygosity were also very low in Uludağ and Ovit Pass populations. However, these values were very high in other populations (Figure 1). Conformably, Gorman et al. (1975) reported that the values of heterozygosity as known genetic variability parameters were lower in island populations of lacertids than in mainland populations. In addition, 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. Similar to their explanation, we found that Uludağ population of *D. b. bithynica* had lower heterozygosity. According to our values of heterozygosity, Ovit Pass population of *D. r. mirabilis* also seems to carry an island population structure. Both populations inhabit montane regions and were isolated from the gene flow. In montane regions, both spatial isolation and differential selection are potentially important factors of speciation (Tarkhnishvili et al. 2013).

Other populations (except from Ovit Pass) of *D. rudis* in the present study have higher heterozygosity as in other

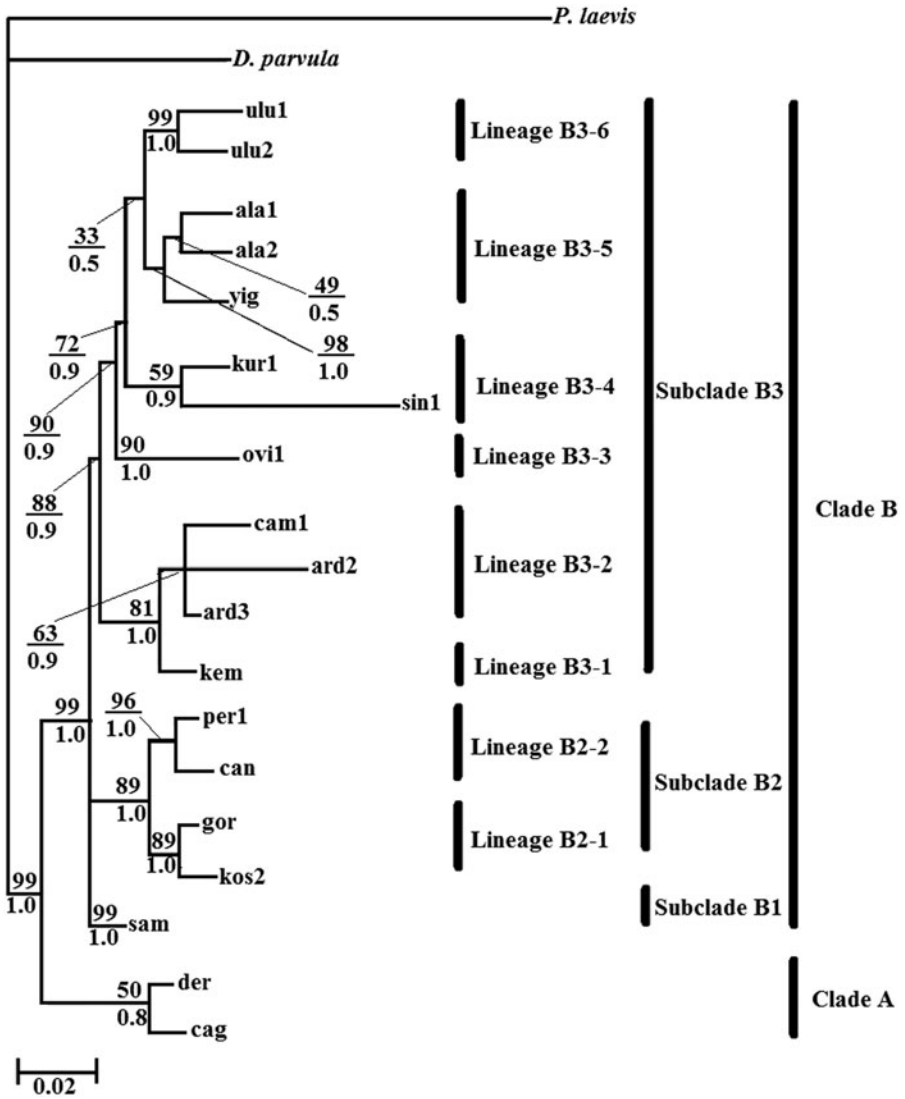

Figure 5. Bayesian tree of a 991-bp sequence of combined data for *D. bithynica* and *D. rudis*. Numbers above branches represent bootstrap support for NJ/ML/MP (1000 replicates) inherence, and numbers below branches indicate Bayesian Posterior Probabilities.

mainland lacertid populations. Similar to our findings, MacCulloch et al. (1995) found that *D. rudis* populations from Achaldaba, Georgia exhibited higher heterozygosity such as mainland lacertid populations.

Genetic distances observed among the Uludağ population of *D. b. bithynica* and *D. b. tristis* populations were very high while they were very low among the populations of *D. b. tristis* and *D. rudis* according to the values of Nei's genetic distance, Nei's unbiased genetic distance, Fst and Linear Fst respectively. Our results showing the genetic proximity between *D. b. tristis* and *D. rudis* are incompatible with the results of Arribas et al. (2013), based on external morphology and osteology of the specimens. On the other hand, the genetic distances among *D. rudis* populations were very low in the present study. Similarly, Ryabinina et al. (2003) revealed the low genetic differences between *D. r. bischoffi* and *D. r. obscura* using RAPD and new inter MIR-PCR method.

Although PCoA analysis (based on values of Fst and Linear Fst) was diverged *D. b. bithynica* population (Uludağ) and *D. r. mirabilis* (Ovit) population from other populations, the values of Nei's genetic distance and Nei's unbiased genetic distance parameters did not diverge these populations from other populations very well. The remaining populations in the present study were close to each other for all parameters.

Mitochondrial DNA

The *p*-distances in 16S rRNA among the Clades (A–G) were very low (Table 4). This could be explained by high levels of gene flow between the respective populations, as implied in the study of Kornilios et al. (2011).

Although Arribas et al. (2013) separated five subspecies (*D. r. rudis*, *D. r. bischoffi*, *D. r. macromaculata*, *D. r. obscura* and *D. r. mirabilis*) of *D. rudis* from Central and Eastern Black Sea), our genetic distances observed among different clades in our samples were very low to distinct these taxa. For example, *p*-distances between Clade A (samples of *D. r. bischoffi*, *D. r. macromaculata* and *D. r. obscura*) and Clade B (*D. r. mirabilis*) was 1.1%; while it was 0.8% between Clade A and Clade C (*D. r. rudis*).

Table 4. Comparison of uncorrected *p*-distance (in %) for fragments of the 16S among nine genetic groups recognized: Clade A, Clade B, Lineage C1, Lineage C2, Lineage C3, Clade D, Clade E, Clade F and Clade G.

		1	2	3	4	5	6	7	8	
No	16S rRNA									
1	Clade A	–								
2	Clade B	1.1	–							
3	Lineage C1	0.7	0.6	–						
4	Lineage C2	0.5	0.6	0.4	–					
5	Lineage C3	1.4	0.6	0.6	0.6	–				
6	Clade D	0.9	0.8	1.0	1.4	1.4	–			
7	Clade E	0.7	1.2	0.8	0.8	0.8	1.0	–		
8	Clade F	1.9	1.0	0.6	0.6	0.6	0.8	0.2	–	
9	Clade G	1.4	0.5	1.5	1.5	1.3	1.7	1.1	0.9	
		1	2	3	4	5	6	7	8	9
No	cytb									
1	Clade A	–								
2	Subclade B1	1.1	–							
3	Lineage B2-1	2.4	1.4	–						
4	Lineage B2-2	3.3	2.3	2.5	–					
5	Lineage B3-1	2.8	1.9	2.8	2.8	–				
6	Lineage B3-2	3.0	2.2	3.0	2.8	1.0	–			
7	Lineage B3-3	3.2	2.9	3.7	4.0	3.1	3.8	–		
8	Lineage B3-4	4.4	3.6	3.8	4.6	3.4	3.8	3.4	–	
9	Lineage B3-5	4.0	3.2	4.0	4.6	3.8	4.2	3.8	3.3	–
10	Lineage B3-6	3.7	2.9	3.5	4.2	3.4	3.6	3.1	2.5	2.5

Comparison of uncorrected p-distance (in %) for fragments of the cytb among ten groups recognized: Clade A, Subclade B1, Lineage B2-1, Lineage B2-2, Lineage B3-1, Lineage B3-2, Lineage B3-3, Lineage B3-4, Lineage B3-5 and Lineage B3-6.

Darevskia 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). However, the *p*-distances in the present study were very low for 16S rRNA among the clades representing *D. rudis* (Clades A-C) and *D. bithynica* (Clades D-G). The *p*-distances among Clades A–C and Clades D–G ranged from 0.5 to 1.9%. The genetic distances into the *D. rudis* samples were also low (0.5–1.4%). According to the current literature, *D. bithynica* has two subspecies (*D. b. bithynica* and *D. b. tristis*). Our genetic distances among samples of *D. b. bithynica* (Clade G) and *D. b. tristis* (Clades D-F) ranged from 0.9 to 1.7%.

The *p*-distances in cytb among the Clades (Clade A and Clade B) were relatively higher than the distances in 16S rRNA (Table 4). The *p*-distances between Clade A (representing der and cag haplotypes of *D. r. rudis*) and Subclade B1 (sam haplotype of *D. r. rudis*) was 1.1%, while it was 2.4% between Clade A and lineage B2-1 (gor and kos2 haplotypes of *D. r. rudis*) and 3.3% between Clade A and B2-2 (per 1 and can haplotypes of *D. r. rudis*). The *p*-distances between Subclade B1 and Lineage B2-1 was 1.4%, while it was 2.3 between Subclade B1 (sam) and Lineage B2-2. Similar to the morphological findings of Arribas et al. (2013), our results showed that *D. r. rudis* was most variable taxon in *D. rudis* species.

The genetic distances between Clade A (der and cag haplotypes of *D. r. rudis*) and Lineage B3-1 (kem haplotype of *D. r. bischoffi*) was 2.8%, while it was 3% between Clade A and Lineage B3-2 (ard 2 and 3 haplotypes of *D. r. obscura* and cam 1 haplotype of *D. r. macromaculata*) and 3.2% between Clade A and Lineage B3-3 (ovi1 haplotype of *D. r. mirabilis*). When compared to the values of the *p*-distances among *D. r. rudis* populations with the other subspecies of *D. rudis*, our results showed that the genetic distances among the nominate and other subspecies of *D. rudis* in the Black Sea were not significantly higher than the distances among the populations of the nominate subspecies. Contrary to the morphological and osteological findings of Arribas et al. (2013), our mtDNA data did not show significant genetic differences among five subspecies of *D. rudis* from Black Sea.

On the other hand, the *p*-distance between Lineage B3-1 (*D. r. bischoffi*) and Lineage B3-2 (*D. r. obscura* and *D. r. macromaculata*) was only 1%. Similar to our findings, Ryabinina et al. (2003) reported that genetic distances between *D. r. bischoffi* and *D. r. obscura* did not exceed 0.8% based on their results of RAPD and new inter-MIR-PCR. These low values lead to a discussion of the validity of these subspecies.

The genetic distances were 3.1% between Lineage B3-1 and Lineage B3-3 (*D. r. mirabilis*) and 3.8% between Lineage B3-2 and Lineage B3-3. When compared with the values of the *p*-distances among *D. r. bischoffi*, *D. r. obscura*, *D. r. macromaculata* and *D. r. mirabilis* populations, it was found that the *p*-distance among *D. r. obscura*, *D. r. macromaculata* and *D. r. mirabilis* was higher than the *p*-distance between *D. r. bischoffi* and *D. r. mirabilis*. Based on the relatively higher *p*-distance (3.8%), our genetic distances in the cytb show that the last described (Arribas et al. 2013) subspecies (*D. r. mirabilis*) in the Black Sea is slightly distinct than *D. r. bischoffi*, *D. r. obscura* and *D. r. macromaculata*.

The genetic distances among the clades representing *D. rudis* and *D. bithynica* for cytb were relatively higher than the distances in 16S rRNA. They ranged from 2.9% (between Subclade B1 representing sam haplotype of *D. r. rudis* and Lineage B3-6 representing ulu1 and ulu2 haplotypes of *D. b. bithynica*) to 4.6% (between Lineage B2-2 representing per1 and can haplotypes of *D. r. rudis* and Lineage B3-4 representing kur1 and sin1 haplotypes of *D. b. tristis*). Consistent with the results of Arribas et al. (2013) stating the existence of two species (*D. rudis* and *D. bithynica*) in Turkey, the *p*-distances among *D. rudis* and *D. bithynica* populations seem to be high.

However, the *p*-distances among the samples of *D. bithynica* were also not low [they ranged from 2.5% (between Lineage B3-4 and Lineage B3-6) to 3.3% (between Lineage B3-4 and Lineage B3-5)].

Strong environmental changes, such as tectonic uplifts (Davis 1971; Kosswig1955; Schmidtler 1998) and Quaternary climatic oscillations (Avise 2000; Hewitt 2001, 2004), might have affected the distribution of the *Darevskia* genus, triggering the evolution and allopatric divergences of various lineages within Anatolia. Particularly, the formation of the Anatolian mountain chains (e.g. the Anatolian Diagonal, the Taurus and the Black Sea Mountains) can be tracked back to the Tertiary, when the northward movement of Europe resulted in the formation of the Alps. During glacial phases, high mountains provided barriers to species dispersion, while during inter-glacial periods, individuals radiating from their refuge often met and promoted secondary contacts and hybridization among their partially distinct lineages (Bellati et al. 2015). Arribas et al. (2013) described a new subspecies (*D. r. mirabilis*) from live high mountainous region (Ovit Pass) in the eastern Black Sea, but they did not study the phylogenetic relationships. Although we did not find a high-level genetic differences at the species level for the specimens of Ovit Pass, the speciation process may have been continued for these lizards.

At the Plio-Pleistocene boundary, wide sea-level changes and extensive tectonic uplifts of land masses, particularly occurring in western and southern Anatolian regions, were the main geological factors affecting taxa distributions (Glover and Robertson 1998). Such orographic and climatic barriers to gene flow have been invoked to explain the great cryptic genetic differentiation recently revealed by molecular studies in several Anatolian taxa (e.g. *Lyciasalamandra* spp., Veith and Steinfartz 2004; *Pelophylax* spp., Akïn et al. 2010; *Typhlops vermicularis*, Kornilios et al. 2011; *Blanus* spp., Sindaco et al. 2014). Consistent with these literatures, Arribas et al. (2013) separated the *D. rudis* complex into the two species (*D. rudis* and *D. bithynica*) based on morphological and osteological data. However, our phylogenetic analyses showed that the samples from western Black Sea (*D. b. tristis*) and from central and eastern Black Sea (*D. r. rudis, D. r. bischoffi*, *D. r. obscura* and *D. r. macromaculata*) were not genetically distinct from each other and *D. rudis* seems to be continuing its status as a complicated species.

Although the multivariate approach (CDA and UPGMA trees derived from distances among populations) of Arribas et al. (2013) clearly showed the differences between *D. bithynica* and *D. rudis*, they also stated that the females of *D. rudis* were not significantly differentiated from those of *D. bithynica* because of the few females in the *D. bithynica* samples. They also did not fully synonimize *D. b. tristis* because it is currently totally allopatric with *D. b. bithynica*, despite the lack of discrimination with *D. b. tristis* and the extremely poor discrimination in ANOVA.

The use of two mitochondrial sequences, even of relatively short length, is generally considered to provide adequate information to point out the occurrence of intra- vs. inter-specific relationships in reptile phylogenies (Poulakakis et al. 2005; Lymberakis et al. 2007; Carranza et al. 2006; Beukema et al. 2010; Rato et al. 2010; Vasconcelos et al. 2010; Bellati 2015). Nevertheless, the phylogenetic relationships among the clades appeared in our study were unresolved, according to the standard mtDNA gene tree approach (NJ, ML, MP and BI).

The *p*-distances in 16S rRNA are low, while it was slightly high in cytb. This gene (cytb) is fast-evolving gene and it may show greater variation than 16S rRNA, which is slow-evolving gene. Therefore, we evaluated the phylogenetic relationships by all parameters (microsatellite DNA and mitochondrial DNA).

In conclusion, microsatellite data [the values of Nei's genetic distance, Nei's unbiased genetic distance (Hedrick 2000), Fst (Hartl & Clark 1997) and Linear Fst (Slatkin 1995) parameters] and similar topologies of the NJ, ML, MP and BI for each gene region and the topologies of ML and BI for combined data showed that *D. rudis* are rather ambiguous, showing a polytomy.

Although microsatellite and mitochondrial DNA markers, that have traditionally been used in phylogenetic and phylogeographic studies (Avise 2000; Rodriguez 2010), might not be sufficient, the mtDNA phylogenetic tree alone might not reflect the true evolutionary history of the species (Zhang & Hewitt 2003; Ballard & Whitlock 2004; Kornilios et al. 2011). Therefore, the phylogenetic relationships should be evaluated based on the nuclear and new mitochondrial markers (longer parts of the16S rRNA and cytb genes or different genes of mtDNA) together to clarify the systematic status of *D. rudis* and *D. bithynica* species from Turkey.

As there are no significant genetic differences among *D. r. obscura*, *D. r. macromaculata* and *D. r. bischoffi*, according to our results and data of Ryabinina et al. (2003), the validity of these taxon as full subspecies should be investigated by comparing Turkish and Georgian samples together, based on the morphological and molecular data. In addition, the phylogenetic relationships among *D. r. bolkardaghica* and the other taxon should be investigated.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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References

Akïn C, Bilgin CC, Beerli P, Westaway R, Ohst T, Litvinchuk SN, Uzzell T, Bilgin M, Hotz H, Guex GD, et al. 2010. Phylogeographic patterns ofgenetic diversity in eastern Mediterranean water frogs were determined by geological processes and climate change in the Late Cenozoic. J Biogeogr. 37:2111–2124.

Arribas O, Ilgaz Ç, Kumlutaş Y, Durmuş SH, Avcï A, Üzüm N. 2013. External morphology and osteology of *Darevskia rudis* (Bedriaga, 1886), with a taxonomic revision of the Pontic and Small-Caucasus populations (Squamata: Lacertidae). Zootaxa. 3626:401–428.

Avise JC. 2000. Phylogeography: The History and Formation of Species. Cambridge, MA: Harvard University Press, p. 447.

Ballard JW, Whitlock MC. 2004. The incomplete natural history of mitochondria. Mol Ecol. 13:729–744.

Başoğlu M, Baran İ. 1977. Türkiye Sürüngenleri: Kaplumbağa ve Kertenkeleler. İzmir, Turkey: İlker Press, p 272.
Bedriaga JV. 1886. Beiträge zur Kenntnis der Lacertiden-Familie. (Lacerta, Algiroides, Tropidosaura, Zerzumia und Bettaia), Abhandlungen der senckenbergischen naturforschenden. Ges Nat. 14:17–443.
Bellati A, Carranza S, Garcia-Porta J, Fasola M, Sindaco R. 2015. Cryptic diversity within the *Anatololacerta* species complex (Squamata: Lacertidae) in the Anatolian Peninsula: evidence from a multi-locus approach. Mol Phylogenet Evol. 82:219–233.
Beukema W, De Pous P, Donaire D, Escoriza D, Bogaerts S, Toxopeus AG, De Bie CAJM, Roca J, Carranza S. 2010. Biogeography and contemporary climatic differentiation among Moroccan *Salamandra algira*. Biol J Linn Soc. 101:626–641.
Bodenheimer FS. 1944. Introduction into the knowledge of the amphibia and reptilia of Turkey. Istanbul Univ Fen Fak Mecm Ser B. 9:1–78.
Boulenger GA. 1904. On the Lacerta depressa of Camerano. Proc Zool Soc. 2:332–339.
Böhme W, Budak A. 1977. Über die rudis-Gruppe des *Lacerta saxicola* Komplexes in der Türkei, I (Reptilia: Sauria: Lacertidae). Salamandra. 13:141–149.
Böhme W, Bischoff W. 1984. *Lacerta rudis* Bedriaga 1886 – Kielschwanz-Felseidechse. In: Böhme W, editor. Handbuch der reptilien und amphibien Europas. Lacerta: Band 2/I Echsen II. p. 332–344.
Budak A, Böhme W. 1978. Über die rudis-Gruppe des Lacerta saaeicola komplexes in der Türkei, I (Reptilia: Sauria: Lacertidae). Ann Naturhistor Mus Wien. 81:273–281.
Carranza S, Harris DJ, Arnold EN, Batista V, Gonzalez de la Vega JP. 2006. Phylogeography of the lacertid lizard, *Psammodromus algirus*, in Iberia andacross the Strait of Gibraltar. J Biogeogr. 33:1279–1288.
Clement M, Posada D, Crandall KA. 2000. TCS: a computer program to estimate gene genealogies. Mol Ecol. 9:1657–1659.
Darevsky IS. 1967. Rock lizards of the Caucasus. (Systematic, ecology and phylogeneis of the polymorphic groups of rock lizards of the subgenus Archaeolacerta). Leningrad: Nauka Press, p. 276.
Davis PH. 1971. Distribution patterns in Anatolia with particular reference to endemism. In: Davis PH, Harper PC, Hedge IC, editors. Plant life of South-West Asia. Edinburgh: The Botanical Society of Edinburgh. p. 15–27.
Farris FS, Kallersjö M, Kluge AG, Bult C. 1995. Constructing a significance test for incongruence. Syst Biol. 44:570–572.
Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution. 39:783–791.
Fu J. 1999. Direct submission.
Fu J, Murphy RV, Darevsky IS. 2000. Divergence of the cytochrome b gene in the *Lacerta raddei* complex and its parthenogenetic daughter species: evidence for recent multiple origins. Copeia. 2:432–440.
Glover CP, Robertson AH. 1998. Role of regional extension and uplift in the Plio-Pleistocene evolution of the Aksu Basin, SW Turkey. J Geol Soc. 155:365–387.
Grechko VV, Bannikova AA, Kosushkin SA, Ryabinina NL, Milto KD, Darevsky IS, Kramerov DA. 2007. Molecular genetic diversification of the lizard complex *Darevskia raddei* (Sauria: Lacertidae): early stages of speciation. Mol Biol. 41:764–775.
Gorman GC, Soule M, Yang SY, Nevo E. 1975. Evolutionary genetics of insular Adriatic lizards. Evolution. 29:52–71.
Guo X, Dai X, Chen D, Papenfuss TJ, Ananjeva NJ, Melnikov DA, Wang Y. 2011. Phylogeny and divergence times of some racerunner lizards (Lacertidae: Eremias) inferred from mitochondrial 16S rRNA gene segments. Mol Phylogenet Evol.61:400–412.
Hartl DL, Clark AG. 1997. Principles of population genetics 3rd ed. Sunderland, MA: Sinauer Associates, Inc.
Hedrick PW. 2000. Genetics of populations. 2nd ed. Boston: Jones and Bartlett.
Hewitt GM. 2001. Speciation, hybrid zones and phylogeography - or seeing genes in space and time. Mol Ecol. 10:537–549.
Hewitt GM. 2004. The structure of biodiversity - insights from molecular phylogeography. Front Zool. 1:4.
Huelsenbeck JP, Hillis DM. 1993. Success of phylogenetic methods in the four- taxon case. Syst Biol. 42:247–264.
Hillis DM. 1987. Molecular vs. morphological approach to systematics. Annu Rev Ecol Syst. 18:23–42.
Jobb G. 2011. TREEFINDER Manual Version of March 2011. distributed by the author, Munich, Germany. Available from: http://www.treefinder.de/ (www.treefinder.de).
Jones DT, Taylor WR, Thornton JM. 1992. The rapid generation of mutation data matrices from protein sequences. Comput Appl Biosci. 8:275–282.
Jukes TH, Cantor CR. 1969. Evolution of protein molecules. In: Munro H, editor. Mammalian protein metabolism. New York: Academic Press, p. 21–132.
Korchagin VI, Badaeva TN, Tokarskaya ON, Martirosyan IA, Darevsky IS, Ryskov AP. 2007. Molecular characterization of allelic variants of $(GATA)_n$ microsatellite loci in parthenogenetic lizards *Darevskia unisexualis* (Lacertidae). Gene. 392:126–133.
Kornilios P, Ilgaz Ç, Kumlutaş Y, Giokas S, Fraguedakis-Tsolis S, Chondropoulos B. 2011. The role of Anatolian refugia in herpetofaunal diversity: an mtDNA analysis of *Typhlops vermicularis* Merrem, 1820 (Squamata, Typhlopidae). Amphibia-Reptilia. 32:351–363.
Kosswig C. 1955. Zoogeography of the Near East. Syst Zool. 4:49–73.
Lantz LA, Cyrén O. 1936. Contribution à la connaissance de *Lacerta saxicola* Eversmann. B Soc Zool Fr. 61:159–181.
Leaché AD, Reeder TW. 2002. Molecular systematics of the eastern fence lizard (*Sceloporus undulatus*): a comparison of parsimony, likelihood, and Bayesian approaches. Syst Biol. 51:44–68.
Lymberakis P, Poulakakis N, Manthalou G, Tsigenopoulos CS, Magoulas A, Mylonas M. 2007. Mitochondrial phylogeography of *Rana* (*Pelophylax*) populations in the Eastern Mediterranean region. Mol Phylogenet Evol. 44:115–125.
MacCulloch RD, Fu J, Darevsky IS, Danielyan FD, Murphy RW. 1995. Allozyme variation in three closely related species of Caucasian rock lizards (Lacerta). Amphibia-Reptilia. 16:1–340.
Méhely LV. 1909. Materialien zu einer Systematik und Phylogenie der muralis-ähnlichen Lacerten. Ann Hist Nat Mus Nat Hun.g 3:409–621.
Milto KD. 2010. Status of the rare rock lizard, Darevskia tristis from pontic mountains. 7th Symposium on the lacertids of the Mediterranean Basin Mallorca, Spain, p 52.
Nei M, Kumar S. 2000. Molecular evolution and phylogenetics. Oxford University Press, New York.
Palumbi SR, Martin A, Romano S, McMillan WO, Stice L, Grabowski G. 1991.The simple fool's guide to PCR version 2.0, privately published document compiled by S. Palumbi. Honululu, Hawaii, HI: University of Hawaii, Deparement of Zoology.
Palumbi SR. 1996. Nucleic acids II: the polymerase chain reaction. In: Hillis DM, Moritz C, Mable BK, editors. Molecular systematics. Sinauer: Sunderland, MA. p. 205–248.
Pavlicev M, Mayer W. 2006. Multiple copies of coding as well as pseudogene c-mos sequence exist in three lacertid species. J Exp Zool B. 306:539–550.
Pavlicev M, Pinsker W, Mayer W. 2011. Phylogenetic relationships among four lizards species of the genus Phoenicolacerta (Squamata: Lacertidae) from the Middle East. Molecular Systematics, Natural History Museum Vienna, Vienna, Austria.
Peakall R, Smouse PE. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. Bioinformatics. 28:2537–2539.
Posada D, Crandall KA. 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics. 14:817–818.
Poulakakis N, Lymberakis P, Antoniou A, Chalkia D, Zouros E, Mylonas M, Valakos E. 2003. Molecular phylogeny and biogeography of the wall-lizard *Podarcis erhardii* (Squamata: Lacertidae). Mol Phylogenet Evol. 28:38–46.
Poulakakis N, Lymberakis P, Valakos E, Pafilis P, Zouros E, Mylonas M. 2005. Phylogeography of Balkan wall lizard (*Podarcis taurica*) and its relatives inferred from mitochondrial DNA sequences. Mol Ecol. 14:2433–2443.
Rato C, Carranza S, Perera A, Carretero MA, Harris DJ. 2010. Conflicting patterns of nucleotide diversity between mtDNA and nDNA in the Moorish gecko, *Tarentola mauritanica*. Mol Phylogenet Evol. 56:962–971.

Rodriguez F. 2010. Integrating phylogeographic patterns of microsatellite and mtDNA divergence to infer the evolutionary history of chamois (genus Rupicapra). BMC Evol Biol. 10:222.

Ronquist A, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 19:1572–1574.

Ryabinina NL, Bannikova AA, Kosushkin SA, Ciobanu DG, Milto KD, Tuniyev BS, Orlova VF, Grechko VV, Darevsky IS. 2003. Estimation of the subspecific level of differentiation in Caucasian Lizards of the Genus Darevskia (SYN. ''*Lacerta saxicola* complex,'' Lacertidae, Sauria) Using Genome DNA Markers. Russ J Herpetol. 9:185–194.

Sindaco R, Kornilios P, Sacchi R, Lymberakis P. 2014. Taxonomic reassessment of *Blanus strauchi* (Bedriaga, 1884) (Squamata: Amphisbaenia: Blanidae), with the description of a new species from south-east Anatolia (Turkey). Zootaxa. 3795:311–326.

Slatkin M. 1995. A measure of population subdivision based on microsatellite allele frequencies. Genetics. 139:457

Schmidtler JF. 1998. Verbreitungsstrukturen der Herpetofauna im Taurus-Gebirge, Türkei (Amphibia; Reptilia). Faunistische Abhandlungen. Staatliches Museum Für Tierkunde in Dresden. 21:133–148.

Swofford DL. 2000. PAUP* Phylogenetic Analysis Using Parsimony (*and Other Methods), version 4. Sunderland, MA: Sinaeur Associates,.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol. 30:2725–2729.

Tarkhnishvili DN, Murtskhvaladze M, Gavashelishvili A. 2013. Speciation in Caucasian lizards: climatic dissimilarity of the habitats is more important than isolation time. Biol J Linnean Soc. 109:876–892.

Tavaré S. 1986. Some probabilistic and statistical problems in the analysis of DNA sequences. Lec Math Life Sci. 17:57–86.

Terent'ev PV, Chernov SA. 1965. Key to the amphibians and reptiles, Isreal Programme of Translations. 3th Edition, Jerusalem, p. 1–315.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24:4876–4882.

Vasconcelos R, Carranza S, Harris DJ. 2010. Insight into an island radiation: the Tarentola geckos of the Cape Verde archipelago. J Biogeogr. 37:1047–1060.

Veith M, Steinfartz S. 2004. When non-monophyly results in taxonomic consequences – the case of *Mertensiella* within the Salamandridae (Amphibia: Urodela). Salamandra. 40:67–80.

Werner F. 1902. Die Reptilien und Amphibienfauna von Kleinasien, SB. Akad. Wiss Wien Math Nat KL. 111:1057–1121.

Zhang DX, Hewitt GM. 2003. Nuclear DNA analyses in genetic studies of populations: practice, problems and prospects. Mol Ecol. 12:563–584.