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Taxonomic Relationships among Turkish Water Frogs as Revealed by Phylogenetic Analyses Using mtDNA Gene Sequences

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We assessed taxonomic relationships among Turkish water frogs through estimation of phylogenetic relationships among 62 adult specimens from 44 distinct populations inhabiting seven main geographical regions of Turkey using 2897 bp sequences of the mitochondrial Cytb, 12S rRNA and 16S rRNA genes with equally-weighted parsimony, likelihood, and Bayesian methods of inference. Monophyletic clade (Clade A) of the northwesternmost (Thrace) samples is identified as *Pelophylax ridibundus*. The other clade (Clade B) consisted of two monophyletic subclades. One of these contains specimens from southernmost populations that are regarded as an unnamed species. The other subclade consists of two lineages, of which one corresponds to *P. caralitanus* and another to *P. bedriagae*. Taxonomic relationships of these two species are discussed and recognition of *P. caralitanus* as a subspecies of *P. bedriagae* is proposed.

Key words: mitochondrial DNA, *Pelophylax ridibundus, Pelophylax bedriagae caralitanus*, *Pelophylax bedriagae*, taxonomy

INTRODUCTION

Turkish water frogs have long been treated as a single species: *Rana ridibunda* (Pallas, 1771) (e.g., Bodenheimer, 1944; Başoğlu and Özeti, 1973). The species, originally described from Gurvev (now Atyrau), Kazakhstan (Dubois and Ohler, 1996 "1994"), occurs very widely from Central Europe, northwards to the Baltic Sea and southwards to the Mediterranean regions, eastwards to Asiatic Russia, and southwards to the Middle East (Frost, 2011). However, Bodenheimer (1944) found specimens with an orange-colored venter from Beyşehir Lake in the Central Anatolia region of Turkey, that were later described as a distinct subspecies *R. ridibunda caralitana* by Arıkan (1988). Distribution area of this subspecies was thereafter widened to the Central Anatolia and Mediterranean regions of Turkey (e.g., Atatür et al., 1990; Ayaz et al., 2006). Joermann et al. (1988) and Schneider et al. (1992) called western Turkey water frogs as *R. levantina*, which name was later replaced by *R. bedriagae* (Dubois and Ohler, 1996 "1994"), although identification of Anatolian water frogs as *R. bedriagae* was challenged by Plötner et al. (2001). Schneider and Sinsch (1999) and Sinsch and Schneider (1999) synonymzed *R. r. caralitana* with *R. bedriagae*, but Jdeidi et al. (2001) insisted that *R. caralitana* is specifically distinct from *R. bedriagae*.

Based on results of molecular phylogenetic studies, Frost et al. (2006) proposed to change the generic name of water frogs from *Rana* to *Pelophylax*. In the most recent list of Turkish water frogs, Franzen et al. (2008) recognized only two species, *P. ridibundus* and *P. caralitanus*, and omitted *R. bedriagae*. Quite recently, Akın et al. (2010a) examined relationships between genetic and morphological characteristics among water frogs from Turkish Lake District. In addition, Akın et al. (2010b) studied detailed genetic variations and estimated the history of diversifications among water frogs from eastern Mediterranean regions including Turkey. However, they did not make any definite conclusions about the taxonomy of these frogs.

In order to understand taxonomic relationships among water frogs within Turkey, we studied samples from across the country using mitochondrial DNA sequence variation. For this purpose we adopted both rapidly (Cytb) and slowly (12S rRNA and 16S rRNA) evolving genes, different from Akın et al. (2010b), who studied only rapidly evolving genes (ND2 and ND3). Finally, we made a taxonomic assessment, rather than divergence time estimation, unlike Akın et al. (2010b).

MATERIALS AND METHODS

Sampling

We examined DNA sequences of Cytb, 12S rRNA, and 16S rRNA genes from 62 adult specimens of 44 distinct populations inhabiting seven main geographical regions of Turkey (Fig. 1, Table 1). Specimen collection was performed in 2007 and 2008. As outgroups, we used sequences of *Pelophylax* (as *Rana*) *nigromaculatus* and *P. chosenicus* from GenBank (Accession Number: NC002805 and EU386874, respectively).

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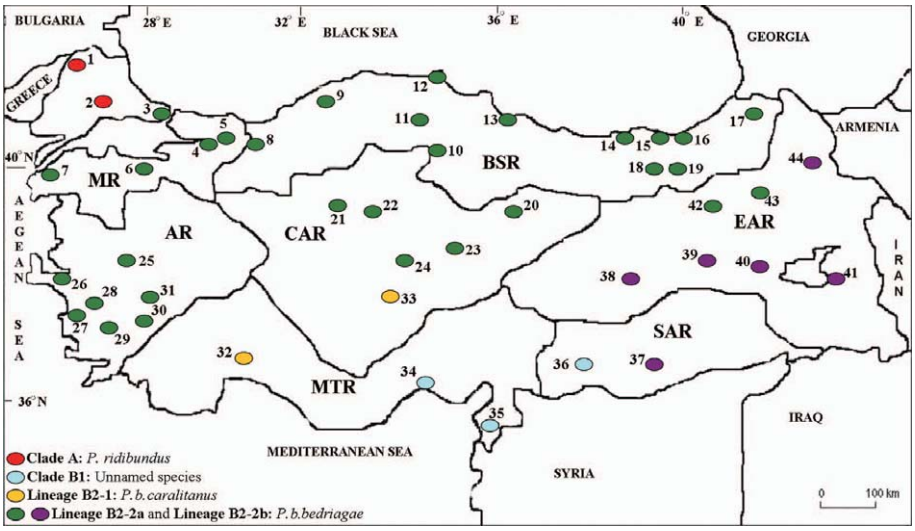

Fig. 1. Map showing the localities of the water frog samples from seven main geographic regions of Turkey. MR: Marmara Region, AR: Aegean Region, MTR: Mediterranean Region, CAR: Central Anatolia Region, BSR: Black Sea Region, EAR: Eastern Anatolia Region, SAR: Southeastern Anatolia Region. For sample numbers, refer to Table 1.

Sequencing

Total DNA was extracted from ethanol-preserved tissues by standard phenol-chloroform extraction (Hillis et al., 1996) or using a commercial kit (Macherey-Nagel, NucleoSpin Tissue Kit, 740952.50) according to the manufacturer's instructions.

A partial sequence of mitochondrial cytochrome b (Cytb), the complete sequences of 12S rRNA, and a partial fragment of 16S rRNA genes were amplified by PCR using the primers listed in Table 2. Cytb amplification involved an initial denaturation step of 7 min at 94°C and 40 cycles of denaturation for 40 s at 94°C, primer annealing for 30 s at 46°C, extension for 60 s at 72°C, and a final 7 min extension at 72°C. The PCR cycle for 12S + 16S rRNA amplification included initial denaturation step of 4 min at 94°C and 33 cycles of denaturation for 30 s at 94°C, primer annealing for 30 s at 53°C, extension for 150 s at 72°C, and a final 7 min extension at 72°C.

Amplified PCR products were purified using polyethylene glycol (PEG) purification procedures; these were used directly as templates for cycle sequencing reactions with fluorescent dye-labeled terminators (ABI Prism Big Dye Terminators v.3.1. cycle sequencing kits). We cycle sequenced the amplified fragments using the primers listed in Table 2. The sequencing reaction products were purified by ethanol precipitation following the manufacturer's protocol and then run on an automated ABI PRISM 3130 genetic analyzer. All samples were sequenced in both directions. The obtained sequences have been deposited in GenBank (AB640897–640996: Table 1).

Phylogenetic analyses

The nucleotide sequences of each gene were aligned using the ClustalW option in the Bioedit software (Hall, 1999). Haplotypes were determined using DAMBE (Xia and Xie, 2001) program. After confirming the suitability for combination of all of the sequences of the three 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 three genes. Phylogenetic analyses based on the combined data were performed by maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) methods. The MP analysis was implemented in MEGA v. 5.01 software package (Tamura et al., 2011) using a heuristic search with the close-neighbor-interchange (CNI) branch-swapping algorithm and ten random-addition replicates. Transitions and transversions were equally weighted, and gaps were treated as missing data. The ML and BI analyses, respectively, were performed using TREEFINDER (Jobb, 2008) and MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). Best fit nucleotide substitution model based on Akaike's information criteria (AIC) was determined for each gene region with KAKUSAN v. 4 software (Tanabe, 2007). In the BI analysis, the following settings were applied: number of Markov chain Monte Carlo (MCMC) generations = six million; sampling frequency = 100; burn-in =10,000. The burn-in size was determined by checking convergences of -log likelihood (-lnL) using Tracer v. l.5 (Rambaut and Drummond, 2007). The robustness of the resultant MP and ML trees were evaluated using non-parametric bootstrap analyses with 1000 and 100 pseudo-replications, respectively, and statistical support of the resultant BI trees was determined based on Bayesian posterior probability (BPP). We a priori regarded tree nodes with bootstrap value (BS) 70% or greater as sufficiently resolved (Huelsenbeck and Hillis, 1993), and those between 50 to 70% as tendencies. In the BI analysis, we considered nodes with a BPP of 95% or greater as significant (Leaché and Reeder, 2002). Uncorrected pairwise sequence divergences for each gene were calculated using MEGA 5.01 v. software package (Tamura et al., 2011).

RESULTS

Sequences and statistics

Sequence statistics for the three gene fragments and the combined alignment, when all nucleotide positions were included, are provided in Table 3. For all 62 ingroup individuals (except for one individual from the GaziAntep population, for which we failed in the PCR amplification of Cytb), we determined sequences of 405 bp of the mitochondrial Cytb, 973 bp of the 12S rRNA, and 1519 bp of the 16S rRNA genes. Of 2897 nucleotides generated, 399 were variable and 331 were parsimony informative. DAMBE program disclosed 20 haplotypes from 61 individuals for Cytb, while 35 haplotypes for 12S rRNA, and 45 haplotypes for 16S rRNA were detected from 62 individuals. Within the ingroup (for 61 individuals), alignment of the combined genes revealed a total of 54 unique haplotypes. Haplotypes were identical between samples 8 and 15; between 13 and 9, 10, 21, and 23; between 28-2 and 30-3; and between 26-1 and 31-1) (Fig. 2).

In the ML analysis, the best fit model selected by KAKUSAN v. 4 software (Tanabe, 2007) for 12S rRNA was J2 (Jobb, 2008) with a gamma shape parameter estimated as 0.227 while it was GTR (Tavaré, 1986) for 16S rRNA and TN93 (Tamura and Nei, 1993) for 1st codon position of Cytb with gamma shape parameters estimated as 0.115 and 9.815, respectively. K80 (Kimura, 1980) and F81 (Felsenstein, 1981) models were selected as the best fit models for 2nd and 3rd codon positions of Cytb, respectively. In the BI analysis, best fit model selected by KAKUSAN for 12S rRNA, 16S rRNA and 1st codon position

Table 1. Samples used for mtDNA analysis in this study together with the information on voucher and GenBank accession numbers. KTUFS = Faculty of Science, Karadeniz Technical University.

Sample No.	Locality	GenBank Accession No.			Voucher
		Cytb	12S rRNA	16S rRNA	
1	Edirne, Büyükdöllük	AB640977	AB640897	AB640932	KTUFS 1
2	Tekirdağ, Malkara	AB640978	AB640897	AB640933	KTUFS 2
3	İstanbul, Şile	AB640986	AB640922	AB640975	KTUFS 3
4	Kocaeli, Şirinköy	AB640986	AB640922	AB640974	KTUFS 4
5	Sakarya, Söğütlü	AB640986	AB640924	AB640973	KTUFS 5
6	Bursa, Nilüfer Brook	AB640986	AB640925	AB640976	KTUFS 6
7	Çanakkale, Kepez	AB640996	AB640926	AB640949	KTUFS 7
8	Bolu, Gerede	AB640986	AB640923	AB640969	KTUFS 8
9	Zonguldak, Çaycuma	AB640986	AB640922	AB640969	KTUFS 9
10	Çorum, Kuşsaray	AB640986	AB640922	AB640969	KTUFS 10
11	Kastamonu, Tosya	AB640986	AB640922	AB640971	KTUFS 11
12	Sinop, Erfelek	AB640986	AB640922	AB640970	KTUFS 12
13	Samsun, Bafra	AB640986	AB640922	AB640969	KTUFS 13
14	Giresun, Piraziz	AB640986	AB640930	AB640969	KTUFS 14
15	Trabzon, Beşikdüzü	AB640986	AB640923	AB640969	KTUFS 15
16	Rize, Fındıklı	AB640986	AB640929	AB640969	KTUFS 16
17	Artvin, Şavşat	AB640995	AB640911	AB640948	KTUFS 17
18	Gümüşhane, Şiran	AB640986	AB640926	AB640969	KTUFS 18
19	Bayburt, Suludere	AB640986	AB640923	AB640968	KTUFS 19
20	Sivas, Serpincik	AB640986	AB640931	AB640969	KTUFS 20
21	Ankara, Ayaş	AB640986	AB640922	AB640969	KTUFS 21
22	Kırıkkale, Bahşılı	AB640986	AB640920	AB640972	KTUFS 22
23	Nevşehir, Gülşehir	AB640986	AB640922	AB640969	KTUFS 23
24-1	Konya, Akşehir Lake	AB640982	AB640901	AB640937	KTUFS 24
24-2	Konya, Akşehir Lake	AB640982	AB640901	AB640952	KTUFS 25
24-3	Konya, Akşehir Lake	AB640983	AB640901	AB640938	KTUFS 26
25-1	Manisa, Karaali	AB640985	AB640903	AB640940	KTUFS 27
25-2	Manisa, Karaali	AB640984	AB640927	AB640954	KTUFS 28
25-3	Manisa, Karaali	AB640986	AB640928	AB640954	KTUFS 29
25-4	Manisa, Karaali	AB640986	AB640920	AB640961	KTUFS 30
25-5	Manisa, Karaali	AB640987	AB640904	AB640941	KTUFS 31
26-1	İzmir, Urla	AB640984	AB640915	AB640954	KTUFS 32
26-2	İzmir, Urla	AB640984	AB640915	AB640962	KTUFS 33
26-3	İzmir, Urla	AB640988	AB640915	AB640962	KTUFS 34
26-4	İzmir, Urla	AB640984	AB640915	AB640963	KTUFS 35
26-5	İzmir, Urla	AB640984	AB640921	AB640964	KTUFS 36
27	Aydın, Söke	AB640984	AB640918	AB640958	KTUFS 37
28-1	Aydın, Germencik	AB640984	AB640917	AB640957	KTUFS 38
28-2	Aydın, Germencik	AB640984	AB640915	AB640939	KTUFS 39
28-3	Aydın, Germencik	AB640984	AB640915	AB640959	KTUFS 40
28-4	Aydın, Germencik	AB640984	AB640919	AB640960	KTUFS 41
29-1	Muğla, Fethiyepaşalı	AB640989	AB640905	AB640942	KTUFS 42
29-2	Muğla, Fethiyepaşalı	AB640990	AB640906	AB640943	KTUFS 43
30-1	Denizli, Kaklık	AB640984	AB640902	AB640939	KTUFS 44
30-2	Denizli, Kaklık	AB640984	AB640916	AB640955	KTUFS 45
30-3	Denizli, Kaklık	AB640984	AB640915	AB640939	KTUFS 46
31-1	Denizli, Acıpayam	AB640984	AB640915	AB640954	KTUFS 47
31-2	Denizli, Acıpayam	AB640984	AB640915	AB640956	KTUFS 48
32	Antalya, Manavgat	AB640980	AB640899	AB640935	KTUFS 49
33-1	Konya, Dineksaray	AB640981	AB640900	AB640936	KTUFS 50
33-2	Konya, Dineksaray	AB640981	AB640914	AB640953	KTUFS 51
34	Mersin, Mezitli	AB640979	AB640898	AB640934	KTUFS 52
35	Hatay, Asi Stream	AB640979	AB640912	AB640950	KTUFS 53
36	Gaziantep, Çaykuyu	–	AB640913	AB640951	KTUFS 54
37	Şanlıurfa, Bozova	AB640991	AB640907	AB640944	KTUFS 55
38	Malatya, Doğanşehir	AB640993	AB640908	AB640966	KTUFS 56
39	Elazığ, Kovancılar	AB640993	AB640908	AB640965	KTUFS 57
40	Bitlis, Adilceviz	AB640993	AB640909	AB640946	KTUFS 58
41	Van, Edremit	AB640994	AB640910	AB640947	KTUFS 59
42	Erzincan, Tercan	AB640986	AB640922	AB640967	KTUFS 60
43	Erzurum, Pasinler	AB640986	AB640926	AB640967	KTUFS 61
44	Kars, Sarıkamış	AB640992	AB640908	AB640945	KTUFS 62

of Cytb was GTR (Tavaré, 1986) with a gamma shape parameter estimated as 6.636, 4.250, and 1.450, respectively. As the best fit models for the 2nd and 3rd codon positions of Cytb, K80 (Kimura, 1980) and F81 (Felsenstein, 1981) models, respectively, were selected. The likelihood value of the ML tree was -lnL = 6336.499.

Phylogenetic relationships

Phylogenetic analyses for the three genes employing three different optimality criteria yielded very slightly different topologies, and only the BI tree is shown in Fig. 2. As shown in the figure, two major clades (Clades A and B) were recovered with strong supports.

The first of these (Clade A) includes northwesternmost (Thrace) samples. Monophyly of the Thrace samples with respect to the other groups was always strongly supported (MP and ML BS = 99 and 100%, respectively; BPP = 100%).

The other clade (Clade B) was also recovered with strong support (MP BS = 97%, ML BS = 96%, BPP = 100%). Clade B consisted of two monophyletic subclades: southernmost subclade (Subclade B1: MP BS = 99%, ML BS = 100%, BPP = 100%) and the other subclade with all the rest of samples (Subclade B2: MP BS = 99%, ML BS = 100%, BPP = 100%).

Subclade B2 consisted of two main lineages: Lineage B2-1, samples from the Central Anatolia and West Mediterranean regions (MP BS = 99%, ML BS = 100%, BPP = 100%), and Lineage B2-2, including all the remaining samples (MP and ML BS both 87%, BPP = 100%). Samples of the Lineage B2-2 were further divided into two sublineages: B2-2b (samples from the Eastern Anatolia region; MP BS = 99%, ML BS = 100%, BPP= 100%) and B2-2a (all the remaining samples; MP BS = 80%, ML BS = 99%, BPP = 100%). Relationships among the samples of B2-2a were unresolved. Uncorrected p-distances among main groups are given in Table 4.

Ventral color variation among lineages

As shown in Fig. 3, samples of the Lineage B2-1 had the characteristically orangish ventral marking which is unique among all lineages.

DISCUSSION

In the most recent reviewing work of water frogs in and around Turkey, Akın et al. (2010b) did not show bases for their identification of samples they studied, or

Table 2. Primers used to amplify mitochondrial DNA in this study.

Target	Name	Sequence 5'-3'	Reference
12S	ThrLm	AAARCATKGGTCTTGTAARCC	Modified from Shaffer and Mcknight (1996)
12S	12SH1	GACACCGTCAAGTCCTTTGGGTTT	This study
12S	L1091	AAACTGGGATTAGATACCCCACTAT	12SA-L in Palumbi et al. (1991)
12S	Hnew	TACCATGTTACGACTTTCCTCTTCT	H1548 in Matsui et al. (2005)
12S	Lnew	TACACACCGCCCGTCACCCTCTT	Shimada et al. (2011)
12S	tval-H	AAGTAGCTCGCTTAGTTTCGG	Shimada et al. (2011)
16S	tval-L	CGTACCTTTTGCATCATGGTC	Shimada et al. (2011)
16S	H2317	TTCTTGTTACTAGTTCTAGCAT	Shimada et al. (2011)
16S	L2204	AAAGTGGGCCTAAAAGCAGCCA	L2188 in Matsui et al. (2006)
16S	Wil6	CCCTCGTGATGCCGTTGATAC	6 in Wilkinson et al. (2002)
16S	L2606	CTGACCGTGCAAAGGTAGCGTAATCACT	16L1 in Hedges (1994)
16S	H3056	CTCCGGTCTGAACTCAGATCACGTAGG	16H1 in Hedges (1994)
Cytb	L14850	TCTCATCCTGATGAAACTTTGGCTC	Tanaka et al. (1994)
Cytb	H15502	GGATTAGCTGGTGTGAAATTGTCTGGG	Tanaka et al. (1994)

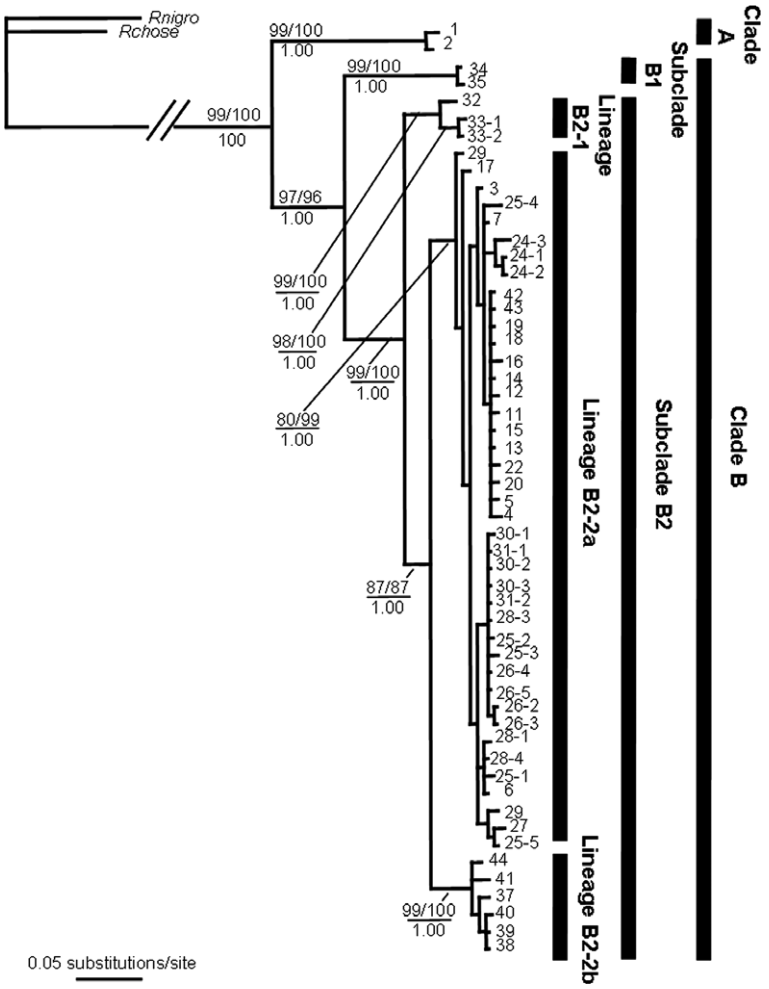

Fig. 2. Bayesian tree of a 2897-bp sequence of Cytb, 12S rRNA, and 16S rRNA for Turkish water frogs. Numbers above branches represent bootstrap support for MP (1000 replicates)/ML (100) inherence, and numbers below branches indicate Bayesian posterior probabilities. For sample numbers, refer to Fig. 1 and Table 1.

make any definite conclusions about taxonomic status of their haplotype groups. Akın et al. (2010b) used only rapidly evolving genes (Mueller, 2006), and with regard to the Turkish samples, they analyzed 340 bp of ND3 genes in 359 individuals and detected 61 haplotypes, and in a longer sequence of 1038 bp of ND2 gene, they found 27 haplotypes in 35 individuals. We used both rapidly and slowly evolving genes and found a slightly smaller amount of divergences (20 haplotypes in Cytb to 45 haplotypes in 16S rRNA) among the Turkish water frogs, probably due to smaller sample size on an average. However, phylogenetic relationships resolved by three different genes we adopted were nearly similar, and resultant combined analysis gave lineages very similar to the haplotype groups reported by Akın et al. (2010b). Thus, our analysis can be regarded as pertinent to discuss taxonomic relationships among the Turkish water frogs.

Table 3. Alignment statistics for fragments of the Cytb, 12SrRNA, and 16S rRNA (all nucleotide positions included); number of base pairs (bp), number of variable sites (vs), number of parsimony informative sites (pi).

	bp	vs	pi
Cytb	405	106	80
12S rRNA	973	109	93
16S rRNA	1519	184	158
Combined	2897	399	331

Our phylogenetic analyses clearly demonstrated the existence of two major monophyletic clades in water frogs from Turkey. One of these (Clade A) includes the northwesternmost (Thrace) samples. From their mt ND2 and ND3 gene sequences data, Akın et al. (2010b) also found frogs from Thrace to constitute a lineage distinct from Anatolian frogs. They identified the frogs from Thrace as *P. ridibundus* because they were very similar to European populations of *P. ridibundus* in sequences. Our analyses using GenBank data also revealed that Clade A forms a monophyletic group with *P. ridibundus* from Greece (DQ474212). Thus, our Clade A should be identified as that species.

Genetic distances observed among different lineages in our samples were not very large, but p-distances in 16S rRNA between Clade A (*P. ridibundus*) and Clade B (≤ 3.1%), are viewed as indicating the level of species difference among the frogs (Fouquet et al., 2007). Thus, the Turkish water frogs in the Clade B are judged to be not conspecific with *P. ridibundus*.

Subclade B1 occupied the most basal position of Clade B while it was judged to be specifically different from Clade A with uncorrected p-distance in 16S rRNA of 3.1%. Although distances among genetic groups in Clade B were not large (≤ 2.3%), distances between Subclade B1 and Subclade B2 (1.9–2.3%) were evidently larger than those between two lineages of Subclade B2 (0.6–1.2%), indicating genetic distinctness of Subclade B1. As discussed below, Subclade B2 itself is considered to contain

two different taxa (Lineage B2-1 [*P. caralitanus*] and Lineage B2-2), and Subclade B1 should better be placed at a higher taxonomic position than these lineages. Unique genetic characteristics of Subclade B1 among Turkish water frogs indicate it to constitute a distinct species for which no available name is found. Frogs in Subclade B1 occur in the southernmost areas of Turkey, and superficially barely distinguishable from frogs of the lineages in Subclade B2, except for Lineage B2-1 (*P. caralitanus*). Further studies including samples from adjacent regions of southernmost Turkey are required to evaluate its taxonomic validity.

Table 4. Comparison of uncorrected p-distances (in %, means followed by ranges in parenthesis) for fragments of the Cytb, 12S rRNA, and 16S rRNA among five genetic groups recognized: Clade A (northwesternmost); Subclade B1 (southernmost); Lineage B2-1 (central Anatolia and western Mediterranean): Lineage B2-2b (eastern Anatolia): Lineage B2-2a (all remaining samples).

	1	2	3	4
Cytb				
1 Clade A	–			
2 Subclade B1	5.3 (5.2–5.4)	–		
3 Lineage B2-1	5.2 (4.9–5.4)	4.0 (4.0–4.2)	–	
4 Lineage B2-2a	5.2 (4.9–5.7)	3.4 (3.2–3.7)	2.0 (1.7–2.5)	–
5 Lineage B2-2b	5.2 (4.9–5.4)	3.9 (3.7–4.2)	2.0 (1.5–2.5)	1.0 (0.5–1.5)
12S rRNA				
1 Clade A	–			
2 Subclade B1	1.9 (1.8–1.9)	–		
3 Lineage B2-1	2.2 (2.2–2.2)	1.2 (1.0–1.3)	–	
4 Lineage B2-2a	2.1 (1.9–2.4)	1.2 (1.0–1.5)	0.6 (0.4–0.9)	–
5 lineage B2-2b	2.7 (2.6–2.8)	1.7 (1.6–1.9)	1.1 81.1–1.3)	0.7 (0.5–0.9)
16S rRNA				
1 Clade A	–			
2 Subclade B1	3.1 (3.1–3.1)	–		
3 Lineage B2-1	2.5 (2.4–2.6)	2.1 (2.0–2.1)	–	
4 Lineage B2-2a	2.8 (2.7–2.9)	2.1 (2.0–2.3)	1.0 (0.8–1.2)	–
5 Lineage B2-2b	2.8 (2.6–2.8)	2.1 (1.9–2.1)	0.8 (0.6–0.9)	0.8 (0.6–1.0)

In Subclade B2, Lineages B2-1 and B2-2 may be considered heterospecific, as they occur nearly sympatrically in the Central Anatolia region. Lineage B2-1 is restricted to the Central Anatolia and Western Mediterranean regions, where *Rana ridibunda caralitana* (= *Pelophylax caralitanus*) was described. Our samples in this clade exhibit orange color and characteristic marking on the ventral surface that fit the original description of *P. caralitanus*. Thus, Lineage B2-1 should be identified as that taxon. Heterospecific relationships of lineages in Clade B and *P. ridibundus* (Clade A) are as discussed above, and recognition of *P. caralitanus* as a species conforms to the conclusion of Jdeidi et al. (2001). However, the fact is not so simple as discussed by Akın et al. (2010a: see below).

Based on bioacoustic data, Schneider and Sinsch (1999) considered water frogs from Beyşehir Lake (type locality of *R. r. caralitanus*) and Agean and Western Mediterranean regions as *R. bedriagae*. Their recognition of *P. bedriagae* and *P. ridibundus* in Turkey conforms to our conclusion, but synonymization of *P. caralitanus* with *P. bedriagae* requires some considerations. Schneider and Sinsch (1999) actually found significantly lower dominant frequency of mating calls in *P. caralitanus* than in other species, but they ascribed this difference to the bigger size of *P. caralitanus*. If indeed the growth pattern such as the size at sexual maturity differs among water frogs, resultant frequency difference in mating calls can be regarded as meaningful in determining species relationships. Thus, the findings of Schneider and Sinsch (1999) may need to be reevaluated. At the same time, because their sampling of calls within Turkey was not necessarily adequate, denser sampling is required to reach more convincing taxonomic conclusion for the water frogs in the country.

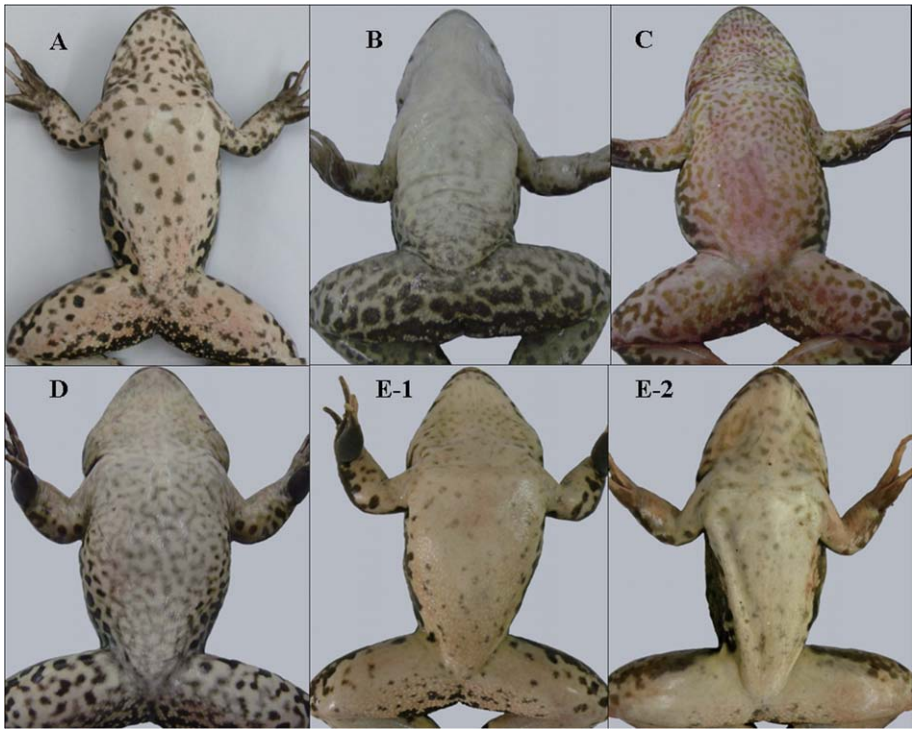

Fig. 3. Ventral color variation among five genetic groups recognized. (**A**) Clade A, (**B**) Subclade B1, (**C**) Lineage B2-1, (**D**) Lineage B2-2a, (**E-1** and **E-2**) Lineage B2-2b. Not to scale.

Sinsch and Schneider (1999) also compared morphological features of *P. caralitanus* with topotypic specimens of *P. ridibundus* and *P. bedriagae* from Kazakhstan and Syria, respectively. They concluded that *P. caralitanus* is conspecific with *P. bedriagae* simply because they considered ventral coloration is generally variable among frogs. In contrast, Jdeidi et al. (2001), by similarly performing morphological comparison of water frogs, but in a wider range of Turkey, recognized distinct specific status of *P. caralitanus* and reported the syntopic occurence of *P. caralitanus* with *P. bedriagae*.

More recently, Akın et al. (2010a)

examined the relationships between mtDNA haplotype and ventral colour in water frogs from localities surrounding the type locality of *R. r. caralitana* (*P. caralitanus*). They found a monophyly of the *caralitana* haplotypes in their phylogenetic tree from short sequences of mitochondrial ND3 gene (340-bp). However, some frogs in this clade did not exhibit orange-colored venters, the unique character of *P. caralitanus.* At the same time, some frogs with non-*caralitana* haplotypes possessed an orange-colored venter. Akın et al. (2010a) considered this discordance in mtDNA haplotype and ventral coloration as a result of unidirectional genetic introgression in periphery regions of Beyşehir Lake, and rejected identification of *P. caralitanus* solely on the basis of ventral colour or mtDNA haplotype. They, however, did not give any conclusive idea about the taxonomic relationships among these frogs, but suggested the necessity of further studies including those on morphometric ratios, mating call parameters, and nuclear markers.

Akın et al. (2010a) used "*Rana* (*ridibunda*) *caralitana*, 1988 Arıkan" in the title of their paper, but this has no taxonomic meaning. From the sampling localities and positions on the phylogenetic tree, *caralitana* and non-*caralitana* lineages in Akın et al. (2010a) clearly correspond to our Lineages B2-1 and B2-2, respectively. If indeed reciprocal genetic introgression through hybridization between the *caralitana* (B2-1) and the non-*caralitana* (B2-2) lineages occurs as suggested by Akın et al. (2010a), these two lineages could be considered as conspecific, but different subspecies, because they consist of interbreeding, basically geographically isolated populations (Mayr and Ashlock, 1991). Because one of two sublineages in Lineage B2-2 (B2-2b) forms a monophyletic clade with *P. bedriagae* from Syria (type locality of the species) in an analysis with the GenBank data of the species (DQ474181), the Lineages B2-1 and B2-2 should be collectively identified as *P. bedriagae*. Thus, we recommend the use of *P. bedriagae caralitanus* instead of *P. caralitanus* for our Lineage B2-1.

Finally, Lineage B2-2 contained two distinct lineages, B2-2a and B2-2b, but they are allopatric and genetically not much divergent (p-distance in 16S rRNA < 1%). We therefore consider them consubspecific at the moment, and call them *P. b. bedriagae*.

In conclusion, we suggest that three distinct species, *P. ridibundus*, unnamed species, and *P. bedriagae* (*P. b. bedriagae* and *P. b. caralitanus*), should be recognized as water frogs of Turkey.

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