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Molecular systematics and phylogeography of *Bufotes variabilis* (syn. *Pseudepidalea variabilis*) (Pallas, 1769) in Turkey

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Abstract: Although there have been several studies based on the molecular data of green toads, some ambiguities related to the systematics of these toads in Turkey still remain. Thus, we used combined mitochondrial genes (D-loop and 12S ribosomal RNA) to resolve these taxonomic problems. We also applied demographic analysis to elucidate the evolutionary history using these genes. We found 3 haplotype groups: 1 for *Bufo viridis* and 2 lineages for *Bufotes variabilis*, with only the latter represented in Turkey. This study showed a genetic diversity within Anatolian *B. variabilis*. Population genetic analysis of mismatch distributions, Tajima's D-statistic, and Fu's Fs test are consistent with a range expansion of the *Bufotes variabilis* group covering western Turkey. *B. variabilis* populations of lineage 1 and lineage 2, from western and eastern Anatolia respectively, are genetically identical. Mitochondrial DNA haplotype groups may reflect historical separation within *B. variabilis*. Our study indicated that allopatric distribution of *B. variabilis* within Anatolia occurred due to climatic shifts during the Pliocene. This study shows that Anatolia likely has served as a factor in vicariant species formation.

Key words: *Bufo* (*Bufotes*) *variabilis*, green toad, 12S rRNA, D-loop, Turkey

1. Introduction

Phylogeography deals with historical phylogenetic components of the geographic distributions of genealogical lineages, particularly those within and among closely associated species (Avise, 2000). Intraspecific phylogeographical patterns usually arise from biogeographic barriers to gene flow like vicariance and dispersal (Avise, 2000; Kornilios et al., 2011). The effects of climatic cycles and vicariance events can possibly be connected to a more cryptic phylogenetic structure (Avise, 2000; Brunsfeld et al., 2001; Bell et al., 2011), whereas demographic events such as population expansion following contraction may leave lasting imprints on phylogenetic structure and genetic variation (Mahoney, 2004).

The taxonomy of the genus *Bufo* has been controversial in the last decade. First, Frost et al. (2006) combined the former "*Bufo*" *viridis* group with a new genus described as *Pseudepidalea* and suggested that *Bufo* must be partitioned into several genera. According to Stöck et al. (2006), *P. variabilis* is distributed from Greece eastwards through Turkey, Cyprus to Syria, Lebanon, and western Saudi Arabia. It is also found in Iraq and Iran and is recorded as being distributed throughout the Caucasus and Russia to Kazakhstan (IUCN, 2013). Dubois and Bour (2010) then showed that *Pseudepidalea* is a junior synonym of *Bufotes* (Rafinesque, 1815). They also recommended 3 distinct subgenera of the single genus *Bufo* (*Bufo*, *Bufotes*, *Epidalea*); therefore, *P. variabilis* was changed to *Bufo* (*Bufotes*) *variabilis* (Pallas, 1769). *Bufo* (*Bufotes*) *variabilis* (Pallas, 1769) (syn. *P. variabilis*) belongs to the family *Bufonidae*, composed of 50 genera with a worldwide distribution except for Australia (http://amphibiaweb.org). Finally, Frost et al. (2013) suggested using *Bufotes variabilis* because of the nonmonophyly of *Bufo*. Therefore, we use *Bufotes variabilis* as the scientific name in this study.

The taxonomy of the green toads in the Middle East including Turkey has been studied morphologically (Eiselt and Schmidtler, 1973; Yılmaz, 1984; Yılmaz and Uğurtaş, 1990; Balletto et al., 1985), by color patterns (Eiselt and Schmidtler, 1973; Yılmaz, 1984; Yılmaz and Uğurtaş, 1990; Balletto et al., 1985; Stöck et al., 2001), osteologically

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(Kete, 1992; Tosunoğlu, 1999), by serological characters (Tosunoğlu, 1999; Borkin et al., 2001; Odierna et al., 2004), and by molecular studies (Stöck et al., 2006; Özdemir and Kutrup, 2007).

However, there has not been a detailed molecular systematic study in Anatolia for *B. variabilis* that examines the presence of a phylogeographic break within the species. The recent studies (Wang, 2009; Murphy et al., 2010; Rogell et al., 2010; Zhan and Fu, 2011; Garcia-Porta et al., 2012; Arntzen et al., 2013) have been published to deal with causes and consequences of vicariance and the evolutionary histories of Bufonidae. Here, we aim to examine the systematic situation and reveal the historical processes that are shaping the biogeography of the species *B. variabilis* by the analysis of mtDNA sequences. Combinations of phylogenetic tests were used to analyze historical events at intraspecific levels and the taxonomy of *B. variabilis*.

2. Materials and methods

2.1. DNA isolation, amplification, and sequencing

A total of 46 green toads were sampled from Turkey, Russia, Greece, Kazakhstan, Azerbaijan, and Albania (Figure 1; Table 1). The animals were treated in accordance with the guidelines of the local ethics committee (Karadeniz Technical University, 2007/12-05). Tissue samples consisted of adult toes stored in 70% ethanol. Genomic DNA was extracted from the toads' toes using the NucleoSpin Tissue Kit (Macherey-Nagel) according to the manufacturer's instructions. We amplified a portion of 868 bp of the mitochondrial control region (D-loop) using primers ControlB-H (5'-GTCCA TTGGA GGTTA AGATC TACCA-3') and CytbA-L (5'-GAATY GGTGG WCAAC CAGTA GAAGA CCC-3'). Polymerase chain reaction (PCR) amplification was done according to procedures described by Stöck et al. (2006) and Goebel et al. (1999). L1091 (5'-AAAAAGCTTCAAACTGGGATTA GATACCCCACTAT-3') and H1478 (5'-TGACTGCAGAG GGTGACGGGCGGTGTGT-3') primers described by Kocher et al. (1989) were used for the 12S gene (423 bp). Purification of PCR products and sequencing was performed by Macrogen, Inc. (Seoul, South Korea). The sequences have been deposited in GenBank (Table 1). Finally, *Bufotes pewzowi* was chosen as the outgroup.

2.2. Phylogenetic analyses and demographic analysis

DNA sequences were aligned using Clustal X (Thompson et al., 1997) and subsequently adjusted by sight. All sequences had the same length and therefore no gaps were postulated.

Phylogenies were reconstructed using the Bayesian inference and maximum likelihood (ML) methods. We carried out Bayesian analyses using MrBayes v. 3.1.2 (Huelsenbeck and Ronquist, 2001) for a given model of sequence evolution. Models of evolution were applied to individual molecular partitions and determined for each gene using MrModeltest v. 2.3 (Nylander, 2004) for

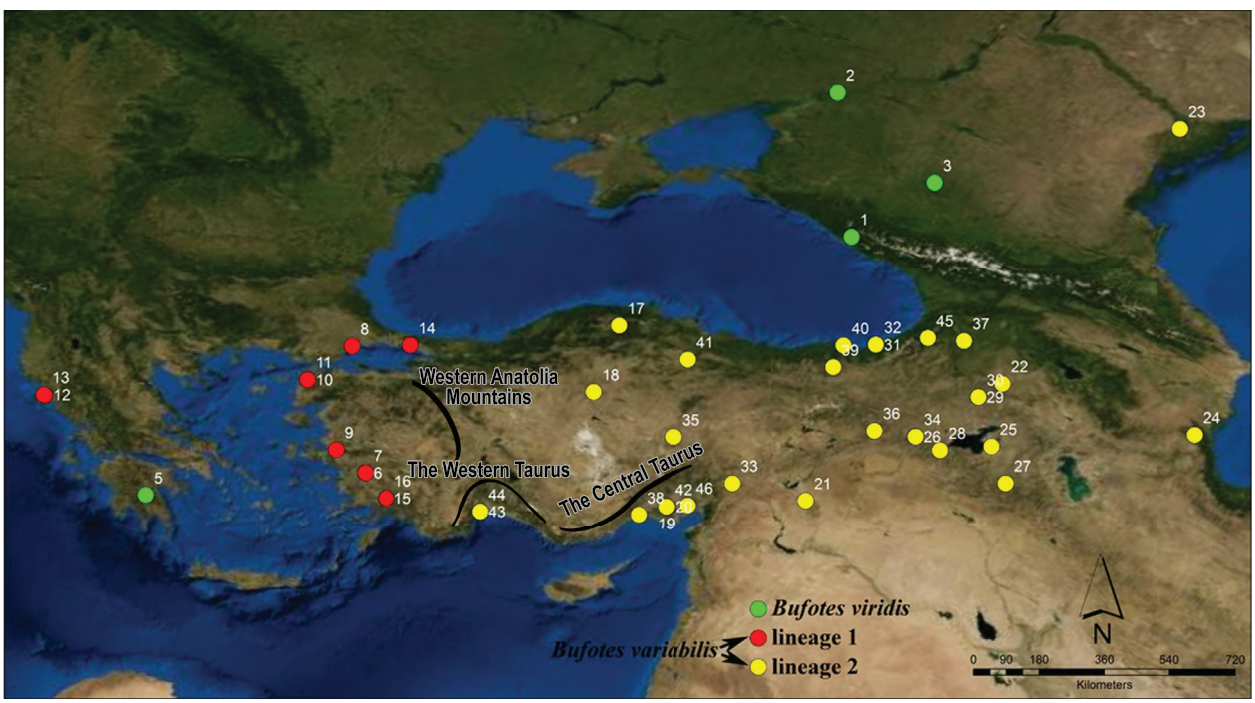

Figure 1. Map of Turkey, Greece, Albania, Russia, and Azerbaijan showing localities of samples sequenced for this study and haplotype group (*Bufotes viridis*, lineage 1, and lineage 2 of *B. variabilis*) assignments for populations based on phylogenetic analyses.

Table 1. *Bufotes viridis*, lineage 1 and lineage 2 of *B. variabilis* sample localities, haplotype group, and GenBank accession numbers. Locality numbers as in Figure 1.

Taxa	Latitude	Longitude	Localities	DNA ID	Map numbers	Haplotype groups	12S	D-loop
B. viridis	43.70574	39.936253	Russia/Krasnodar, Solokh-Aul	BV5	1	BV5	GQ489031	GQ489073
B. viridis	47.265786	39.590602	Russia/Rostov	BV7	2	BV7	GQ489033	GQ489074
B. viridis	45.03913	41.98214	Russia/Stavropol	BV14	3	BV5	GQ489036	GQ489078
B. viridis	55.75579	37.61763	Russia/Moscow	BV56	4	BV5	GQ489060	GQ489100
B. viridis	37.29325	22.42915	Greece/Peloponessos, Argos	BV8	5	BV8	GQ489034	GQ489075
B. variabilis	37.8444	27.8458	Turkey/Aydın	BV19	6	BV19	GQ489032	GQ489080
B. variabilis	37.84368	27.84966	Turkey/Aydın	BV37	7	BV19	GQ489033	GQ489074
B. variabilis	40.98333	27.51667	Turkey/Tekirdağ, Çorlu	BV51	8	BV19	GQ489034	GQ489075
B. variabilis	38.41885	27.12872	Turkey/İzmir, Çiğli	BV55	9	BV55	GQ489059	GQ489099
B. variabilis	40.15531	26.41416	Turkey/Çanakkale	BV59	10	BV19	GQ489062	GQ489101
B. variabilis	40.15211	26.41491	Turkey/Çanakkale, Gelibolu	BV84	11	BV19	JX439777	JX439766
B. variabilis	39.759849	19.944091	Albania/Sarande	BV65	12	BV65	GQ489068	GQ489105
B. variabilis	39.7799	19.9141	Albania/Sarande	BV64	13	BV19	GQ489067	GQ489104
B. variabilis	41.00527	28.97696	Turkey/İstanbul	BV81	14	BV19	JX439774	JX439762
B. variabilis	37.21528	28.36361	Turkey/Muğla	BV82	15	BV82	JX439775	JX439764
B. variabilis	37.21447	28.36876	Turkey/Muğla	BV83	16	BV19	JX439776	JX439765
B. variabilis	41.494072	34.144182	Turkey/Kastamonu	BV18	17	BV18	GQ489038	GQ489079
B. variabilis	39.84682	33.51525	Turkey/Kırıkkale	BV36	18	BV18	GQ489048	GQ489089
B. variabilis	37	35.32133	Turkey/Adana, Yüreğir	BV85	19	BV85	JX439778	JX439767
B. variabilis	36.99844	35.32551	Turkey/Hatay, Dörtyol	BV86	20	BV86	JX439779	JX439763
B. variabilis	37.15	38.8	Turkey/Sanlıurfa, Ceylanpınar	BV9	21	BV9	-	JX439769
B. variabilis	40.049477	43.654287	Turkey/Iğdır, Tuzluca	BV3	22	BV3	GQ489029	GQ489071
B. variabilis	46.35489	48.05272	Russia/Astrakhan	BV12	23	BV3	GQ489035	GQ489076
B. variabilis	38.77528	48.41528	Azerbaijan/Lerik	BV15	24	BV15	GQ489037	GQ489078
B. variabilis	38.49417	43.38	Turkey/Van	BV23	25	BV23	GQ489040	GQ489081
B. variabilis	38.74329	41.50648	Turkey/Muş	BV24	26	BV24	GQ489041	GQ489082
B. variabilis	37.58333	43.73333	Turkey/Hakkari, Beytüşşebap	BV25	27	BV25	GQ489042	GQ489083
B. variabilis	38.4	42.11667	Turkey/Bitlis	BV29	28	BV29	GQ489045	GQ489086
B. variabilis	39.72167	43.05667	Turkey/Ağrı	BV26	29	BV26	GQ489043	GQ489084
B. variabilis	39.72066	43.06057	Turkey/Ağrı	BV27	30	BV27	GQ489044	GQ489085
B. variabilis	41.02005	40.52345	Turkey/Rize	BV30	31	BV30	GQ489046	GQ489087
B. variabilis	41.02006	40.52788	Turkey/Rize	BV31	32	BV30	GQ489047	GQ489088
B. variabilis	37.58333	36.93333	Turkey/Kahramanmaraş	BV33	33	BV30	JX439780	JX439770
B. variabilis	38.74177	41.50978	Turkey/Muş	BV45	34	BV24	GQ489053	GQ489093
B. variabilis	38.73333	35.48333	Turkey/Kayseri	BV43	35	BV43	GQ489051	GQ489092
B. variabilis	38.88535	40.49829	Turkey/Bingöl	BV40	36	BV29	GQ489050	GQ489091
B. variabilis	41.11048	42.70217	Turkey/Ardahan	BV48	37	BV48	GQ489055	GQ489094
B. variabilis	36.8	34.63333	Turkey/Mersin	BV46	38	BV46	GQ489054	JX439771
B. variabilis	40.46028	39.48139	Turkey/Gümüşhane	BV50	39	BV30	GQ489056	GQ489095
B. variabilis	41	39.73333	Turkey/Trabzon	BV52	40	BV29	GQ489058	GQ489097
B. variabilis	40.65	35.83333	Turkey/Amasya	BV57	41	BV57	GQ489061	JX439772
B. variabilis	36.99926	35.31658	Turkey/between Adana and Ceyhan	BV60	42	BV60	GQ489063	GQ489102
B. variabilis	36.88414	30.70563	Turkey/Antalya, Serik	BV66	43	BV66	GQ489069	GQ489106
B. variabilis	36.88099	30.70747	Turkey/Antalya, Serik	BV67	44	BV66	GQ489070	GQ489107
B. variabilis	41.18333	41.81667	Turkey/Artvin	BV69	45	BV69	JX439781	JX439773
B. variabilis	37.0317	35.82275	Turkey/ between Adana and Ceyhan	BV61	46	BV60	GQ489064	GQ489103

Bayesian analyses. The best evolution model for each partition was selected using the Akaike information criterion (Akaike, 1974) in MrModeltest. These analyses showed that the best-fitting models were GTR for 12S and GTR+I+gamma for D-loop. For the combined mtDNA analysis, 2 replicate searches were carried out for 2.0×10^6 generations. We examined stationary plots of the log probability of the data during running. Sample trees were generated before likelihood values reached stationary; they were discarded as burn-in values. The ML analyses were performed using the program RAxML v. 7.0.3 (Stamatakis, 2006). GTR (General Time Reversible) model parameters of nucleotide substitution with the Γ model of rate heterogeneity were estimated by RAxML. Two hundred inferences were executed using RAxML, and nonparametric bootstrap proportions with 1000 replicates were used for estimating nodal support.

Phylogenetic analysis showed that there are 3 haplotype groups (Figure 1). They were addressed as distinguishing units to examine regional genetic variety between and within populations. For comparisons of genetic diversity within the region, nucleotide diversity (p) and haplotype diversity (h) were estimated using Arlequin v. 3.1 (Excoffier et al., 2006). Diversification between populations and within species based on haplotype frequencies was calculated by Markov chain methodology (10,000 steps, 1000 dememorization steps). In addition, we evaluated Tajima's D (Tajima, 1989) and executed Fst values using Arlequin v. 3.1 (Excoffier et al., 2006) in order to examine the hypothesis of demographic expansion. Graphs of pairwise differences among sequences were generated using DNAsp v. 5 (Librado and Rozas, 2009). The frequency distribution of the number of pairwise differences among all sequences (mismatch distribution) was generated for all samples in the region. If a population shows unimodal mismatch distribution in haplotype networks, that population has a star-like phylogeny due to the accumulation of low-frequency mutations. However, the population has a long-term demographic stability in multimodal mismatch distribution. Bimodal or multimodal mismatch distribution indicates diminishing population sizes or structured size (Slatkin and Hudson, 1991; Rogers and Harpending, 1992; Schneider and Excoffier, 1999).

3. Results

A total of 868 homologous base pairs of the mitochondrial control region (D-loop) and 423 bp of 12S rRNA were obtained from all specimens. Sequence alignment was straightforward; no insertions or deletions were observed. A total of 28 haplotypes from D-loop and 7 haplotypes from 12S rRNA were identified among the 46 individuals (Table 1). The phylogenetic analyses showed similar topology for both gene regions, with most nodes strongly supported. Since both markers used here are mtDNA markers, it was easy to combine them together within each individual to perform phylogenetic analyses. Therefore, a single tree was produced based on Bayesian and ML methods (Figure 2).

3.1. Phylogenetic analyses

The monophyly of *Bufotes* was strongly supported by the results of the analyses of the combined mitochondrial genes. We identified 2 clades for the *Bufotes* specimens (Figure 2). Clade 1 was detected in Greece and Russia and was identified as *B. viridis*. Clade 2 was detected in Turkey, Albania, Russia, and Azerbaijan. Furthermore, Clade 2 was divided into 2 main lineages: lineage 1 and lineage 2. Lineage 1 had strong support from likelihood bootstrap values (>70%) and Bayesian posterior probabilities (>0.95%), whereas lineage 2 was weakly supported in combined mitochondrial genes (Figure 2). While lineage 1 was found only in western Anatolia, lineage 2 was found in all eastern locations, including Russia (to the north of the Caspian Sea) and Azerbaijan (Figure 1).

3.2. Genetic diversity and regional demographic analyses

We used D-loop and 12S rRNA gene regions for the population genetic structure of the genus *Bufotes* in Turkey. In the D-loop gene region, the pairwise Fst values for the 3 haplotype groups of D-loop sequences are 0.255, 0.583, and 0.594, respectively (Table 2). Nucleotide diversity (π) and haplotype diversity (h) are lower in lineage 1 than in lineage 2 and *B. viridis* (Table 3). Nucleotide differences within haplotypes are highest in lineage 2 (0.139%), followed by the *B. viridis* group (0.04%). The lineage 1 group showed the lowest nucleotide differences (0.009%). Although nucleotide differences between lineage 1 and *B. viridis* (0.558) were similar to those between lineage 2 and *B. viridis* (0.505), nucleotide differences between lineage 1 and lineage 2 were 0.181 (Table 2). Fu's Fs test was not significant for any geographic region ($P > 0.05$), while Tajima's D was significant only in the lineage 1 group ($P = 0.018$). Lineage 2 and *B. viridis* had multimodal distributions (Figure 3a and 3b), while Lineage 1 had a unimodal mismatch distribution for the D-loop gene analyzed (Figure 3c). Lineage 1 was significantly different from the null expectation in the D-loop gene region analyzed ($P = 0.00$; Table 3; Figure 3). On the contrary, both *B. viridis* and lineage 1 showed that there are no polymorphisms in the 12S gene region; however, Fu's Fs test of selective neutrality was only significant for lineage 2 ($P = 0.024$; Table 3).

4. Discussion

Our study was about both the genetic analysis and the patterns of distribution of green toads in Turkey. We found 2 main clades among populations of green toads. The first clade was identified as *B. viridis* and the second clade

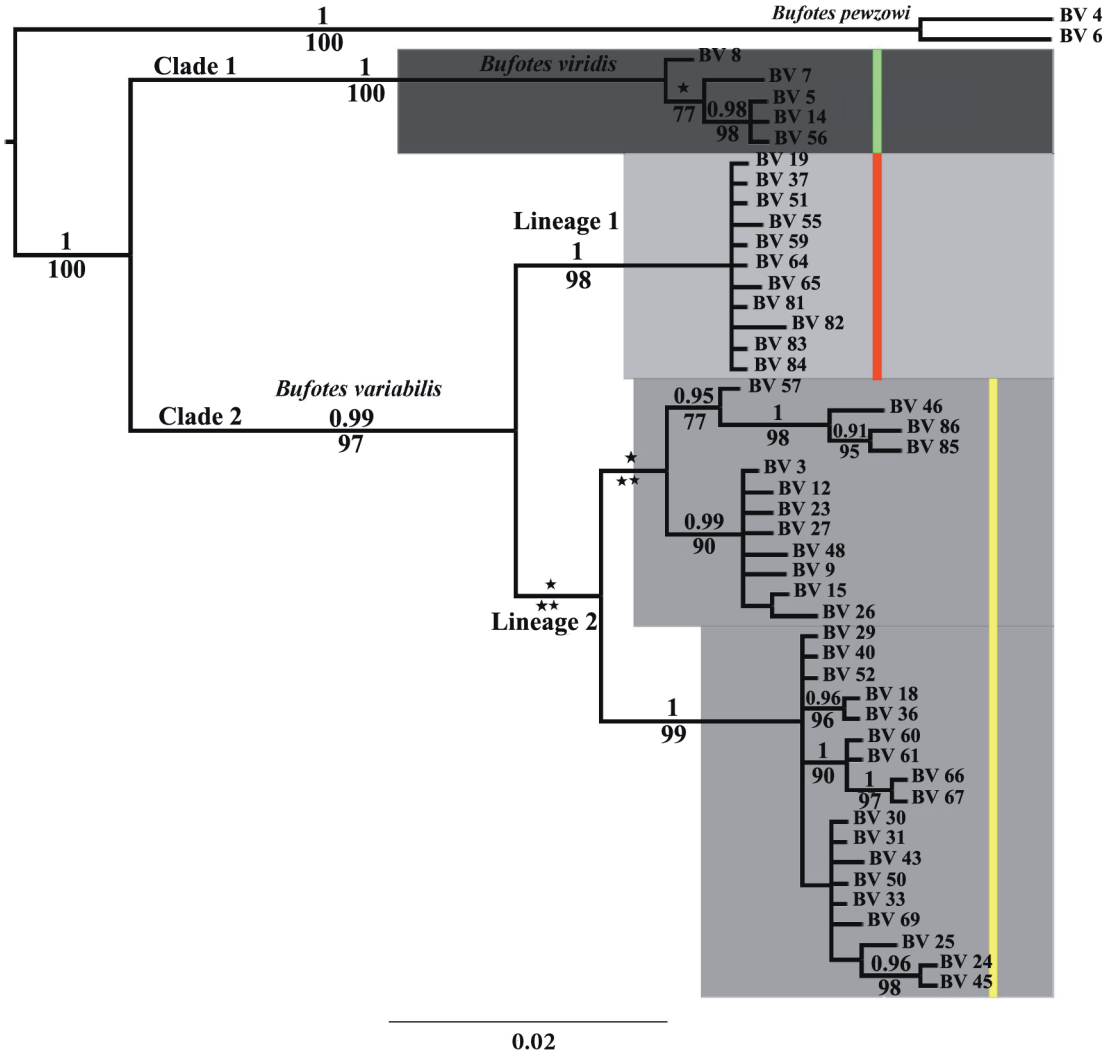

Figure 2. Phylogeny of *Bufotes* populations in Turkey, Greece, Albania, Russia, and Azerbaijan based on Bayesian and ML analysis of the combined mitochondrial genes (D-loop and 12S). Numbers above branches are Bayesian posterior probabilities, and numbers below are likelihood bootstrap support values. Single and double asterisks indicate nodes that are not strongly supported in both Bayesian and likelihood analysis (bootstrap values of <70% and Bayesian posterior probabilities of <0.95). IDs in front of the branches show DNA ID in Table 1.

was recognized as *B. variabilis*. In Turkey, 2 lineages of *B. variabilis* could be distinguished. The first lineage only occurs in western Anatolia, and the second lineage occurs in all eastern regions (Figure 1).

According to Fu's Fs, demographic analyses solely indicate evidence of both population expansions and historical constrictions for lineage 2 using the 12S rRNA gene. On the contrary, lineage 1 of *B. variabilis* follows population expansion models according to Tajima's D-statistic (Table 3). In addition, lineage 1 of *B. variabilis* is significantly different from the null expectation for d-loop gene region. Hence, we focused on the D-loop gene for the demographic analyses.

Table 2. Pairwise differences and Fst values between haplotype groups for the D-loop region. Lower diagonal: Corrected average pairwise differences in percent (PiXY – (PiX + PiY)/2). P-value for all corrected PiXY is 0. Diagonal elements: Pairwise differences within population (PiX). Upper diagonal: Fst values between haplotype groups.

	B. viridis	Lineage 1	Lineage 2
B. viridis	0.04	0.583	0.594
Lineage 1	0.558	0.009	0.255
Lineage 2	0.505	0.181	0.139

Table 3. Results of demographic parameters including h, p, Fu's Fs, Tajima's D, and SSD (95% CI and P-values given where applicable), grouped by geographic region.

Haplotype groups	*n*	n_h	Haplotype diversity (*h*)	Nucleotide diversity (π)	Fu's *Fs*	Prob (sim. Fs ≤ obs. Fs)	Tajima's *D*	P (D simul < D obs)	SSD (P-value)
D-loop									
B. viridis	5	3	0.70	0.0046	1.87	0.81	−0.33	0.47	0.22 (0.07)
Lineage 1	11	4	0.49	0.00105	−0.94	0.14	−1.79	0.018	0.26 (0.00)
Lineage 2	30	21	0.96	0.0161	−2.65	0.19	0.35	0.69	0.027 (0.41)
12S									
B. viridis	5	1	0.00	0.00	0.00	-	0.00	1.00	-
Lineage 1	11	1	0.00	0.00	0.00	-	0.00	1.00	-
Lineage 2	30	4	0.306	0.00075	−2.21	0.024	−1.35	0.072	0.0076 (0.37)

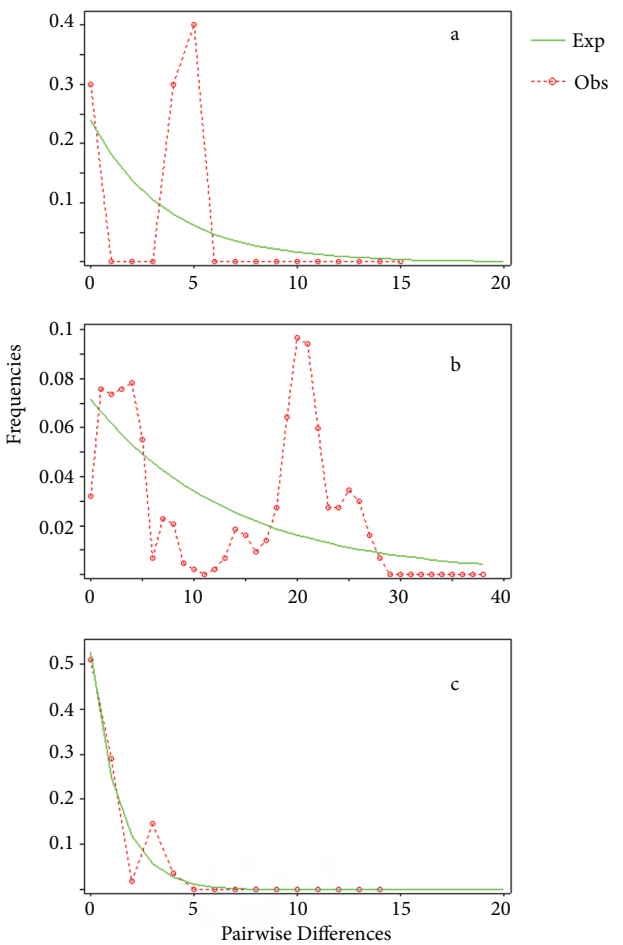

Figure 3. Mismatch distributions for different population subsets of genus *Bufotes* compared to the expected frequencies under the demographic expansion model (a = *B. viridis*, b = lineage 1 of *B. variabilis*, c = lineage 2 of *B. variabilis*).

Our results showed the presence of a genetic break across Anatolia. This is probably due to the Anti-Taurus curving northeast from the Taurus (Toros) and western Anatolian Mountains, a well-known biogeographical hotspot for amphibians and reptiles (Schmidtler, 1998), which also showed a strongly supported and divergent sublineage of lineage 2 in this study (BV46, 85–86) (Figure 1). In addition, the causes of more sublineages of lineage 2 were explained by Anatolian mountain ranges (especially the Anatolian Diagonal), because Anatolia was exposed to more geological events during the glacial period. Therefore, Anatolian mountain ranges were major barriers for the sublineage dispersal of lineage 2. Similarly, Kornilios et al. (2011) revealed 4 well-supported lineages for *Typhlops vermicularis* within their sampled populations, which correspond to respective refugia within Anatolia. They stated that the Anatolian peninsula is a predominantly mountainous area whose diverse geomorphology produces many different climatic regions and vegetation types. In addition, Akın et al. (2010) indicated that 2 clades of water frogs may have split from the uplift of the Taurus Mountains, and they also showed phylogeographic patterns of genetic diversity for water frogs as determined by geological processes and climate in Anatolia. During the Tertiary and Quaternary, Anatolia acted either as a bridge or as a barrier for species dispersal between Asia and Europe, providing a natural pathway or acting as a vicariant agent (Tchernov, 1992). Repeated temperature fluctuations during these periods pushed Anatolian populations from south to north and vice versa (Çıplak, 2003). All these features render Anatolia a biologically diverse region that has played an important role in producing and sustaining animal and plant diversity. The substantial patterns of genetic variation and demographic features within Anatolia are a reflection of the complex

history of the region. The phylogeographic break within *B. variabilis* probably reflects results from the glacial history of the region, and this may also apply to the break between *B. viridis* in the Balkans and the Anatolian *Bufotes* (Greece, loc. 5; Albania, loc. 12–13).

Differences between Balkan and Anatolian individuals can chiefly be connected with the formation of the Aegean in the late Pliocene. The genetic pattern of the green toads observed in Turkey suggests that populations were isolated during the ice ages and subsequently differentiated genetically. Here, our results clearly confirm a genetic barrier between populations of *B. variabilis* based on locations relative to the Anti-Taurus and western Anatolian Mountains, suggesting a causal role for the genetic variation of *B. variabilis* to the western Taurus Mountains, whose altitude varies between 3000 and 3750 m a.s.l. This was probably the result of their proximity to the Mediterranean Sea and the favorable humid climatic conditions during the Last Glacial Maximum (Sarıkaya et al., 2008). In addition, the divergent lineages of *B. variabilis* in the east and the west are allopatric distributional within Anatolia. This result is consistent with another genetic study (Özdemir and Kutrup, 2007) based on mitochondrial data from the 16S rRNA gene indicating the differentiation of Tekirdağ and İzmir populations from the rest of Turkey.

Stöck et al. (2006) studied green toads from the entire Palearctic range (including Turkey, from 9 localities) by using phylogenetic and demographic methods. They studied the control regions that reveal 12 haplotype groups. They found only 1 haplotype group (2n-VI) in Anatolia that occurs also in Cyprus, the Middle East, western Iran, the Caucasus, the steppes of northwestern Kazakhstan, and north of the Aral Sea, and then also in Scandinavia, in Germany (type locality), and in Greece. As a result of that study, these authors tentatively refer to these populations as *B. variabilis* (Pallas, 1769), since their range included the type locality. In addition, Stöck et al. (2006) stated that the possible contact zone of *B. variabilis* and *B. viridis* is in Greece, because both *B. viridis* (Peloponnese, Alepochori, Lake Nemea, and Crete) and *B. variabilis* (Peloponnese and Patra) were found in Greece.

As a result, it was suggested that the populations in Turkey can be regarded as lineage 2, except for the western populations that were attributed to lineage 1. Our results support the idea of a possible *B. variabilis*/*B. viridis* contact zone in Greece as suggested by Stöck et al. (2006). Further sampling across Greece and East Europe is needed to fully clarify genetic variation and subspecies status within the species in this area.

Acknowledgments

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