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# Intraspecific variation of Turkish Green Toads, *Bufo* (*Pseudepidalea*) *viridis* LAURENTI, 1768, based on 16S ribosomal RNA sequences

(Anura: Bufonidae)

## Innerartliche Variabilität türkischer Wechselkröten, *Bufo* (*Pseudepidalea*) *viridis* LAURENTI, 1768, anhand von 16S rRNS Sequenzen

(Anura: Bufonidae)

NURHAYAT ÖZDEMİR & BİLAL KUTRUP

KURZFASSUNG

Vierzehn Individuen von *Bufo* (*Pseudepidalea*) *viridis* LAURENTI, 1768 aus 14 türkischen Populationen wurden hinsichtlich der Variabiltät ihrer mitochondrialen 16S rRNS untersucht. Dabei ließen sich 10 Haplotypen nachweisen. Paarweise Vergleiche der DNS-Sequenzen dieser Haplotypen zeigten Unterschiede von bis zu 12 Basensubstitutionen; die Sequenzunterschiede betragen 0,11 % bis 1,28 %. Die Unterschiede gegenüber Individuen aus Armenien und der Ukraine haben ein ähnliches Ausmaß (0,11 % bis 1,08 %). Das geschätzte Divergenzalter der verschiedenen mtDNS Haplotypen legt ihre Pleistozene Differenzierung vor etwa 0,78 Millionen Jahren nahe. Maximum parsimony, maximum likelihood, Bayes- und TCS Analysen zeigten, daß es bei türkischen Wechselkröten eine geographische Differenzierung in zwei Gruppen gibt: die westlichen Populationen (İzmir, Tekirdağ) einschließlich der ukrainischen Tiere ('*viridis*') stehen allen übrigen türkischen Wechselkröten einschließlich derer aus Armenien ('*variabilis*') gegenüber. Es bedarf jedoch noch weiterer Untersuchungen, um die genetische Vielfalt innerhalb dieser Art zu erklären.

ABSTRACT

Fourteen individuals of *Bufo* (*Pseudepidalea*) *viridis* LAURENTI, 1768 representing 14 populations from Turkey were examined for variation in the mitochondrial 16S rRNA. Ten haplotypes were recovered. Pairwise comparisons of DNA sequences among haplotypes showed differences of up to 12 base substitutions; sequence divergences ranged from 0.11 % to 1.28 %. The divergence to individuals from Armenia and Ukraine was similar (0.11 % to 1.08 %). The divergence time estimated between several mtDNA haplotypes suggested a Pleistocene differentiation approximately 0.78 million years ago. Maximum parsimony, maximum likelihood, Bayesian and TCS analyses show that, there is geographic substructuring in Turkish Green Toads in that they are represented by two clades in the study area: western populations (İzmir, Tekirdağ) including the Ukraine ('*viridis*') versus all others from the rest of Turkey including Armenia ('*variabilis*'). Further investigations are needed to better understand the genetic variation within the species.

KEY WORDS

Amphibia: Anura: Bufonidae: *Pseudepidalea viridis*, *Bufo viridis*, green toad, 16S rRNA, phylogeny, systematics, taxonomy, molecular biology, Turkey, Caucasus, Asia Minor

## INTRODUCTION

The Green Toad, *Bufo viridis* LAURENTI, 1768 (only recently placed into the Genus *Pseudepidalea* by FROST et al. 2006), is distributed over a very large range that covers much of Europe except France and the Iberian Peninsula, North Africa, the Arabian Peninsula, and Asia as far east as China and Mongolia (GIACOMA et al. 1997). This broad area of distribution is characterized by ecological conditions, from coastal environments to mountains (over 2000 m elevation), and from desert to riparian forest. In Turkey, the Green Toad was reported primarily from lowland habitats (from sea level to 300-500 m; EISELT 1965; CLARK & CLARK 1973; YILMAZ & UĞURTAŞ 1990; TOSUNOĞLU 1996; TOK 1999) but also from Ardahan, Bayburt and Gümüşhane (1380-1312 m; KUTRUP et al. 2006a).

The taxonomy of the Green Toad in Turkey has been confusing. Until recently, Green Toads were thought to form just one

subspecies, *Bufo viridis viridis*, distributed in the lowland area of Turkey (TERENTJEV & CHERNOV 1949; MERTENS & WERMUTH 1960; EISELT 1965). FLINDT & HEMMER (1968) considered the specimens from Adana-Hatay (Southern Anatolia) as *B. viridis arabicus* MERTENS, 1967. Later, the taxonomy and nomenclature of *Bufo arabicus* was extensively analyzed by BALLETTO et al. (1985) who restricted the name after examination of the type to a separate species from the Arabian Peninsula (SW Saudi Arabia, N-Oman, E-Emirates). Although *B. arabicus* was not considered to belong to the *B. viridis* group, but to a separate species group, the name *arabicus* was repeatedly used in a subspecific combination by different authors to term green toads from Middle East, Iran, Pakistan and even India (STÖCK et al. 2001).

Several morphological characters and differing colour patterns have been used by authors in distinguishing subspecies and species in Turkey. In morphological and serological characters of toads from western and southern Anatolia, TOSUNOĞLU (1996) found that *B. viridis arabicus* from Adana, was not significantly different from *Bufo viridis viridis* of İzmir. TOSUNOĞLU (1996) also pointed out that specimens from Adana expressed similar morphological characters and colour patterns when compared to those from the Black Sea lowland region. In contrast, TOK (1999) stated that the specimens inhabiting Datça Peninsula differ from those of İzmir, Trakya (Thrace) and the Black Sea region by having a thicker first finger; additionally, the dorsal pattern of specimens from Datça shows more similarity to those of Adana-Hatay than to those of any other region. Subsequently, karyological studies of the relationships among populations varying in ploidy levels (BORKIN et al. 2000; ODIERNA et al. 2004) demonstrated that Green Toads from Adapazarı and Ardahan did not show any differences from the other populations of Central Asia, Israel and Europe.

Systematic relationships and speciation patterns within taxa of the *Bufo viridis* complex are long debated issues. In addition, molecular data from Turkish Green Toads is lacking. Recently, STÖCK et al. (2006) studied Green Toads from the entire Palearctic range (including nine localities from Turkey) applying phylogenetic and demographic methods. The authors analyzed control regions that characterize a deeply branched assemblage of twelve haplotype groups, diverged since the Lower Miocene. Only one haplotype group (2n-VI) was found in Anatolia; it also occurs on Cyprus, in the Middle East, Western Iran, Caucasus, in the steppe zone of northwestern Kazakhstan, northern Aral Sea and further east. As a result of the study, the authors tentatively refer to these populations as *B.* (*Pseudepidalea*) *variabilis* (PALLAS, 1769), since their range includes the type locality. BATISTA et al. (2006) analyzed 35 individuals of *B. viridis* including widespread populations for partial mitochondrial 12S and 16S rRNA. Three divergent lineages were determined in this study; one in North Africa and Sicily, another in Europe (including 3 individuals from Turkey), and a third in Sardinia and Mallorca.

Therefore, the validity of the subspecies is not clear and the aim of this study is to identify the taxonomic status of *B. viridis* in Turkey. We sequenced 936 nucleotides of the mitochondrial 16S ribosomal RNA gene to examine the genetic differences; data of *B. viridis* from Vedi, Armenia (AF160797; LIU et al. 2000) and Kiev region, Ukrainian Republic (AY680267; PAULY et al. 2004) were included in the analysis in which *Bufo* (*Epidalea*) *calamita* LAURENTI, 1768 (AF350434; HARRIS 2001) and *Bufo bufo* LINNAEUS, 1758 (AY840 247; KUTRUP et al. 2006b) formed the outgroup.

## MATERIALS AND METHODS

Fourteen *Bufo viridis* individuals were sampled from 14 different locations (Fig. 1, Table 1). They were collected from the provinces of Artvin, Rize, Trabzon, Giresun, Giresun Island, Gümüşhane, Ardahan, Van, Bingöl, Kayseri, Mersin, Hatay, Tekirdağ and İzmir (Turkey) (Table 1).

Tissue samples consisted of adult toes stored in 70% ethanol. Total genomic DNA was extracted using the Qiagen™ method,

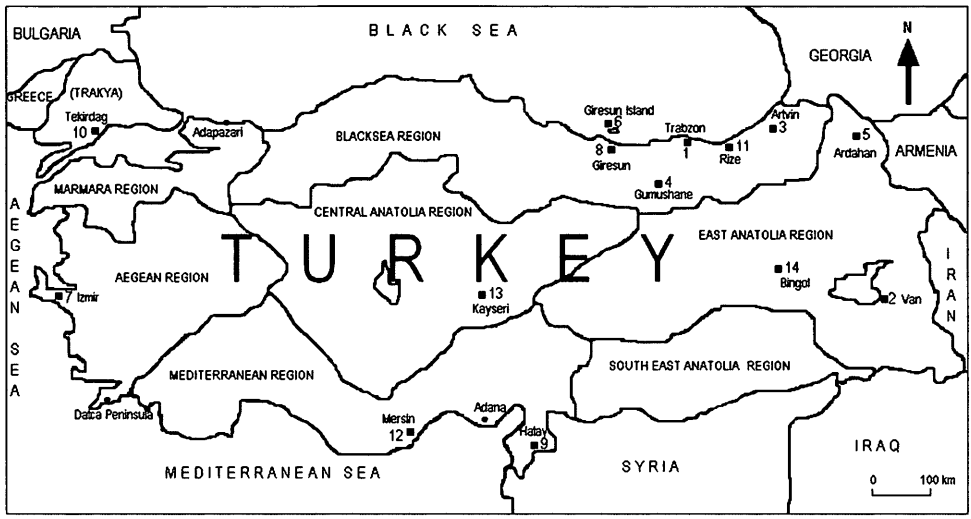


Fig. 1: Localities of the Turkish *Bufo viridis* specimens sampled in this study. Numbers correspond to population/locality numbers listed in Table 1.

Abb. 1: Die Fundorte der in dieser Untersuchung gesammelten türkischen *Bufo viridis* Exemplare. Die Ziffern entsprechen den Populations-/Fundortnummern in Tabelle 1.

following the manufacturer's instructions. We amplified a 936 base pair fragment of the mitochondrial 16S ribosomal RNA gene using primers 16L10 (5' – AGT GGG CCT AAA AGC AGC CA – 3') and 16H1 (5' – CTC CGG TCT GAA CTC AGA TCA CGT AGG – 3') (HAY et al. 1995). PCR amplification was done according to procedures described by HEDGES et al. (1991). The amplified 16S rRNA gene was cloned to pGEM-T vector (a TA clone vector) and sequenced commercially using the universal M13 primers. GenBank accession numbers are AY862555-AY862560 and AY970641-AY970648 (Table 1).

Sequences were aligned using Clustal X (THOMPSON et al. 1997); Modeltest 3.6 (POSADA & CRANDALL 1998) and PAUP* 4.0b10 software (SWOFFORD 2000) were used to choose the model of evolution. Pairwise distances were estimated using the GTR+G model. We employed multiple complementary methods of data analysis: maximum parsimony and maximum likelihood phylogenetic estimation using PAUP* 4.0b10 software (SWOFFORD 2000), Bayesian estimates of relationships using MrBayes 3.0b4 (HUELSENBECK & RONQUIST 2001), and TCS analysis (CLEMENT et al. 2000) to resolve genealogical relationships within *B. viridis*.

Parsimony analyses were performed using heuristic search method (10,000 random addition replicates, tree-bisection-reconnection (TBR) branch swapping) and bootstrap analysis (FELSENSTEIN 1985) was conducted with 100 replicates. The ML tree was then estimated (heuristic search with 10,000 replicates), and bootstrapping (100 replicates) was used to assess support for internal nodes. Bayesian analyses were conducted with MrBayes 3.0b4 (HUELSENBECK & RONQUIST 2001), for a given model of sequence evolution. The GTR+G model was used; model parameters were estimated by MrBayes. Four Markov chains were used in each replicate, and the chain was sampled every 100 generations. Analyses were allowed to run for 2 million generations, discarding 2000 samples as burn-in.

Intraspecific differentiation within *B. viridis* was assessed using unrooted cladograms based on a statistical parsimony procedure (TCS) that has been shown to have greater statistical power and accuracy when there are few variable sites (TEMPLETON et al. 1992; POSADA & CRANDALL 2001). Se-

Table 1: List of *Bufo viridis* samples from Turkey used in this study and mitochondrial haplotypes with accession numbers. One individual per population (locality) was sampled.

Tab. 1: Die in der Untersuchung verwendeten *Bufo viridis* Proben aus der Türkei, die mitochondrialen Haplotypen sowie die GenBank Zugangsnummern. Ein Exemplar pro Population (Fundort) wurde untersucht.

| Population No. | Locality<br>Fundort | Haplotype<br>Haplotyp | GenBank Accession number<br>GenBank Zugangsnummer |
|---|---|---|---|
| 1 | Trabzon | TRA | AY970641 |
| 2 | Van | VAN | AY970642 |
| 3 | Artvin | ART | AY970643 |
| 4 | Gümüşhane | GUM | AY970644 |
| 5 | Ardahan | RIZ | AY970645 |
| 6 | Giresun Island | GIR | AY970646 |
| 7 | İzmir | IZM | AY970647 |
| 8 | Giresun | GIR | AY970648 |
| 9 | Hatay | HAT | AY862555 |
| 10 | Tekirdağ | TEK | AY862556 |
| 11 | Rize | RIZ | AY862557 |
| 12 | Mersin | MER | AY862558 |
| 13 | Kayseri | RIZ | AY862559 |
| 14 | Bingöl | RIZ | AY862560 |

quences were connected under the 95% probability of parsimony criterion using the software TCS (version 1.18, CLEMENT et al. 2000) except the Trabzon haplotype which was omitted from TCS and distance analyses. The resulting network represents the reconstructed gene genealogy of the haplotypes.

## RESULTS

A total of 936 homologous base pairs of the 16S rRNA sequences were obtained in all specimens (except Trabzon specimens that is 550 bp). GenBank accession numbers: AY862555-AY862560 and AY970641-AY970648. Sequence alignment was straightforward, no insertions or deletions were observed. A total of 10 mitochondrial haplotypes were identified among the 14 individuals (Table 1). The sequence divergences calculated according to the GTR+G model among our 10 haplotypes ranged from 0.11 % to 1.28 % and the sequence differences from Armenian and Ukrainian *B. viridis* to our Turkish ones ranged between 0.11 % (İzmir-Ukraine) and 1.08 % (Tekirdağ-Armenia). The Armenian sample differed from our samples by 0.51 % (Van) to 1.08 % (Tekirdağ) and the Ukrainian by 0.11 % (İzmir) to 1.01 % (Van). Populations from Turkey were closely related to those from Europe and Armenia. For 16S rRNA sequences, haplotypes connected by ≤12 substitutions have at least a probability of 0.95 of being parsimoniously connected and a network was generated using TCS (CLEMENT et al. 2000), and all the haplotypes could be joined in a single network (Fig. 2).

The best fitting evolutionary model for our data set found by Modeltest 3.6 was the GTR+G model that had the best likelihood score (-lnL = 2015). Parameters estimates were: base frequencies 0.3355 (A), 0.2261 (C), 0.1805 (G), 0.2579 (T), a Ti/Tv ratio of 2.6558. The parsimony analysis generated fourteen most parsimonious trees. Trees generated from ML and MP analyses approximately showed the same topology. So the ML tree is shown with bootstrap values (Fig. 3). Also the majority rule consensus tree of Bayesian analyses with posterior probability values showed the same topology.

Maximum parsimony, maximum likelihood, Bayesian and TCS analyses show that, there is some geographic substructuring in Turkish Green Toads. One of two distinct clades was found in western Turkey (Tekirdağ, İzmir) including the Ukraine while the other clade was represented all over the rest of Turkey, including Armenia.

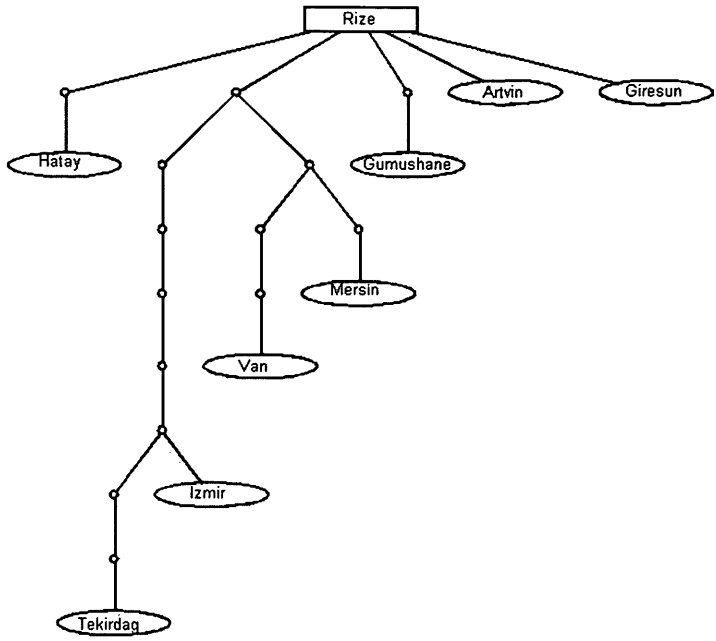


Fig. 2: Single most parsimonious network of relationships among the partial 16S rRNA gene sequences for Turkish *Bufo viridis*. Circles along the branches indicate nucleotide changes, the square on top indicates the presumed ancestral haplotype. The Trabzon haplotype was not considered.

Abb. 2: Einziges sparsamstes (most parsimonious) Modell des Beziehungsnetzes zwischen den 16S rRNS Gen-Teilsequenzen türkischer *Bufo viridis*. Kreise im Astverlauf zeigen Nukleotidwechsel an, das Quadrat am oberen Bildrand bezeichnet den vermuteten anzestralen Haplotyp. Der Trabzon-Haplotyp wurde nicht berücksichtigt.

## DISCUSSION

Partial sequences of 16S rRNA have been widely used in the assessment of relationships within and between amphibian genera. In Asia, LIU et al. (2000) found five variable sites in widely distributed populations of *Bufo* (*Duttaphrynus*) *melanostictus* SCHNEIDER, 1799. HARRIS (2001) reported up to four substitutions from eight populations of *Bufo* (*Epidalea*) *calamita* LAURENTI, 1768 from Western Central Europe, including Britain, examining a 576 bp region of the 16S rRNA gene.

Pairwise comparisons of DNA sequences among *Bufo viridis* populations show differences of up to 12 base substitutions (936 bp) which is not similar to *Bufo gargarizans* CANTOR, 1842 [syn. *B. andrewsi* HU, JIANG & TIAN, 1984] populations studied by MACEY et al. (1998) in Asia (22 base substitutions in 1063 bp). The sequence divergence of our *B. viridis* samples including the ones from Armenia and Ukraine ranged from 0.11 % to 1.28 %. So it was found that there is low variation despite a broad geographic range and, these findings are in line with the studies of LIU et al. (2000), HARRIS (2001) and KUTRUP et al. (2006a). In contrary, BATISTA et al. (2006) suggest the presence of three divergent lineages separated by 2.8 – 3.2 % for 12S and 16S rRNA genes in 35 *Bufo viridis* individuals including widespread populations. As the authors said, this is higher than the typical intraspecific variation in Asian bufonids and it is also higher than our results. These authors also found high variation within the European lineage (including 3 indivuals from Turkey) representing evidence of considerable gene flow, with individuals from Turkey, Greece and the Ukraine sharing haplotypes. This is in

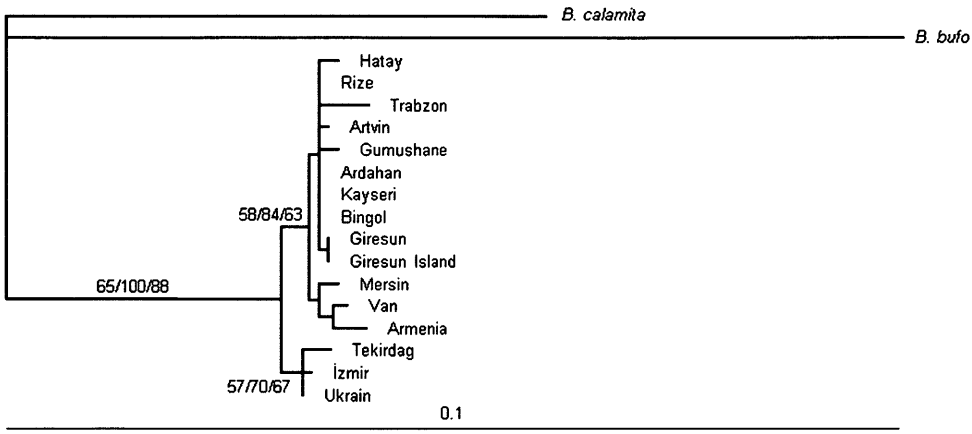


Fig. 3: The maximum likelihood (ML) phylogeny (-lnL = 2015) estimated under the GTR+G model of sequence evolution. Bootstrap (MP) and Bayesian posterior probability values (>50) are indicated on nodes of the tree (ML / MP / Bayesian, respectively). Geographic regions outlined in figure 1.

Abb. 3: Wahrscheinlichste (maximum likelihood, ML) Phylogenie (-lnL = 2015) auf Basis des GTR+G Modells der Sequenzevolution. Bootstrapwerte (MP) und nachträglch ermittelte Wahrscheinlichkeitswerte (>50) der Bayes-Analyse sind an Verzweigungspunkten angegeben (ML / MP / Bayes). Zur Lage der Fundorte siehe Abbildung 1.

line with our results that we found low genetic difference between Turkish and Ukrainian Green Toads.

A molecular evolutionary rate of 0.69 % change per lineage per million years is estimated by MACEY et al. (1998) in the collective Genus *Bufo*. The highest pairwise sequence divergence between *Bufo viridis* populations is 1.08 %. Applying the pairwise rate of 1.38 % (0.69 % per lineage) change per million years, it was calculated to have diverged approximately 0.78 MY BP suggesting late Pleistocene differentiation. The Anatolian plateau uplifted 5 to 10 MYBP due to acceleration of northward movement of the Arabian Plate (QUENNEL 1984; STEININGER & RÖGL 1984). The closing of the Tethys Sea caused periodic isolation of Anatolia from central Europe (STEININGER & RÖGL 1984). Some Palearctic regions were particularly important as corridors for invasions. Anatolia was repeatedly affected by fauna and flora exchanges: it is situated at the interface of the European, Asian, and African biomes from where it experienced repeated invasions since the Late Oligocene (STEININGER et al. 1985; KOSWIG 1955). Therefore, multiple connections between Anatolia and Europe followed by vicariant separations may be expected.

Our data support the existence of genetically differentiated entities, in contrast to the other studies using morphology, serologic differences and chromosomes of Central Asian, European, and North African populations of *Bufo viridis* (TOSUNOĞLU 1996; ODIERNA et al. 2004). All chromatinic markers showed the same pattern and composition in all specimens, independently of their origin and ploidy levels. Also genomic southern hybridizations of pBv to *Pst* I - digested DNA revealed that all the Green Toad populations examined (one of which is from Ardahan, Turkey) shared the same hybridization pattern, regardless of origin and ploidy level (ODIERNA et al. 2004).

BORKIN et al. (2000) examined 15 specimens from western Turkey (Adapazarı) and northern and southern Iran (Tehran and Kerman), for external characters, geographic position, and nuclear DNA content. And the researchers described the sample from Adapazarı as *Bufo viridis viridis*. FLINDT & HEMMER (1968) recognized Green Toads of the Near East as a distinct subspecies, *B. v. arabicus* which should occur, at least, in south-eastern Turkey (Adana). In this study,

we included specimens from Mersin and Hatay (southern Turkey) and contrary to FLINDT & HEMMER (1968) and KETE (1992), we did not find the southern samples to be distinctly different from the main bulk while the western samples were. From two distinct clades in the ML tree, one occurs in western Turkey (Tekirdağ, İzmir), including the Ukraine and the other clade all over the rest of Turkey, including Armenia. In the study by STÖCK et al. (2006), Ukrainian and Greek samples jointly belonged to one haplotype group found outside of Turkey, called 2n-VII and representing *B. viridis viridis*. Our results cluster Tekirdağ and İzmir with Ukrainian specimens suggesting that the western Turkish populations may be referred to as *B. viridis viridis*. Similar to our results, STÖCK et al. (2006) assigned their only population from western Turkey (Muğla) to *B. viridis* ssp., differentiating it from their other Turkish samples that were marked as *Bufo variabilis*. These authors suggested that a contemporaneous event was responsible for initial vicariance of 2n-VI (Asia Minor, Middle East, northern Eurasia) and 2n-VII (Central and southeastern Europe, nothern Asia) clades. So we tentatively concluded that western populations may represent *Bufo viridis viridis* and all those in the rest of Turkey *B. viridis variabilis*, being fully aware of the fact that this latter assignment is not well in accordance with other analyses on the status of the controversially seen taxon *B. variabilis* (comp. comments in FROST et. al. 2006). However, futher sampling across the range of *B. viridis* is needed to better understand genetic variation, taxonomic subdivisions within species, clade boundaries and contact zones.

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AUTHORS: Nurhayat ÖZDEMİR, Rize University, Faculty of Arts and Sciences, Department of Biology, 53100, Rize, Turkey < nurhayat61@yahoo.com >; Bilal KUTRUP, Karadeniz Technical University, Faculty of Arts and Sciences, Department of Biology, 61080, Trabzon, Turkey