Intraspecific variation of *Bufo bufo*, based on 16S Ribosomal RNA sequences

Bilal Kutrup[1], Nurhayat Yilmaz[2,4], Sabriye Canakci[1], Ali Osman Belduz[1], Stefano Doglio[3]

1. Introduction

More than half of the species within the family Bufonidae are contained within the genus *Bufo*. The 450 species of this genus are distributed throughout most major land masses of the world, including the Americas, Eurasia, Africa and excluding Australo-Papuan Realm and Madagascar (Frost, 2002). Because of their cosmopolitan distribution and variable life histories, these toads are of interest to investigators researching evolutionary history and biogeography (Pramuk et al., 2001).

The Common toad *Bufo bufo* is a wide-ranging species, with a distribution encompassing much of Europe (except in northern Scandinavia), parts of northern Africa and western Asia (Lüscher et al., 2001). Three subspecies are recognised: *B. b. gredosicola* from central Spain, *B. b. spinosus* from the Mediterranean region including southern Spain, southern France, southern Switzerland, Italy, several southeastern countries and northern Africa, and *B. b. bufo* from the other parts of Europe, including the north of the Swiss Alps (Lüscher et al., 2001).

The taxonomic status of *Bufo bufo* in Turkey is still disputed. It is believed to be represented by 3 subspecies. Bodenheimer (1944) identified specimens from northwest Anatolia, Aegean Region and Black Sea Region as the nominal subspecies. *Bufo b. verrucosissimus,* distributed in the Caucasies (Orlova and Tuniyev, 1989), was described from Trabzon by Eiselt (1965), who also identified an individual in the Frankfurt Zoology Museum which was caught from Rize as *B. b. spinosus*. Clark and Clark (1973) identified two specimens as *B. b. spinosus* from Hopa (northeast coast of Turkey) and Balikesir (northwest of Turkey). *Bufo b. spinosus* was described from Trakya by Yılmaz (1984), from Datca by Tok (1999), and from Camlıhemsin (Rize) by Baran et al. (1997). Yilmaz and Kumlutas (1995) studied the taxonomic status of *B. bufo* in Turkey and found no significant morphological differences among the Black Sea, Aegean and Mediterranean Regions.

The morphological and genetic differentiation of the *Bufo bufo* group from Europe and Asia has been studied by some authors (Hemelaar, 1988; Reading, 1990; Macey et al., 1998; Lüscher et al., 2001; Pramuk et al., 2001). However molecular data concerning *Bufo bufo* from Turkey is lacking. Therefore the validity of the subspecies are not clear and the aim of this study is to identify the taxonomic status of this species in this region and to examine genetic variation to compare with other taxa for which data have already been published. 936 nucleotides of the mitochondrial 16S ribosomal RNA gene were sequenced to examine the genetic differences, with 890 nucleotides of *B. bufo* (AY325988; Pauly et al., 2004) from Latvian Republic (USSR) also included.

1 - Karadeniz Technical University, Faculty of Arts and Sciences, Department of Biology, 61080 Trabzon, Turkey
2 - Karadeniz Technical University, Faculty of Rize Arts and Sciences, Department of Biology, 53100 Rize, Turkey
3 - Universita di Torino, Biologia Animalia, Via Accademia Albertina, 10123 Torino, Italy
4 - Corresponding author, e-mail: nurhayat@ktu.edu.tr

© Koninklijke Brill NV, Leiden, 2006.
Also available online - www.brill.nl

Amphibia-Reptilia 27 (2006): 268-273



Figure 1. All localities of *Bufo bufo* sampled in this study. Numbers correspond to locality numbers listed in table 1.

Material and methods

A total of 29 *Bufo bufo* individuals were sampled from 16 locations (fig. 1, table 1). They were collected from Erenkoy, Borcka in province of Artvin; from Camlihemsin, Findikli, Karasu, Gundogdu in Rize; from Arakli, Akcaabat, Yesilova, Sumela (Macka) in Trabzon; from Giresun and from Mezitli in Mersin. Three populations were from Europe, including one population from Garaguso, in the province of Matera (Italy) and another from the north shore of the Aoou Lake near Metsova in the Pindos Mountains (Greece). Additional *B. b. spinosus* specimen originated from Sicily (Italy).

Tissue samples consisted of tadpoles or adult toes stored in 70% ethanol. Total genomic DNA was extracted using the Wizard Genomic DNA Purification System (Promega) and Qiagen^{TM}, following the manufacturers instructions. The polymerase chain reaction (PCR) was used to amplify 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 using the universal M13 primers at Davis Sequencing and Macrogen. GenBank accession numbers are AY555020-AY555025 and AY840225-AY840247.

Sequences were aligned using Clustal X (Thompson et al., 1997); there were no indels. Intraspecific differentiation within *Bufo bufo* was assessed using intraspecific unrooted cladograms based on a statistical parsimony procedure that has been shown to have greater statistical power and accuracy when there are few variable sites (Templeton et al., 1992; Posada and Crandall, 2001). Sequences were connected under the 95% probability of parsimony criterion using the software TCS (version 1.18, Clement et al., 2000). The resulting network represents the reconstructed gene genealogy of the haplotypes.

2. Results

A total of 936 homologous base pairs of the 16S rRNA sequences were obtained in all specimens (GenBank accession numbers: AY555020-AY555025 and AY840225-AY840247). A total of 23 mitochondrial haplotypes were identified among the 29 individuals examined (table 1). The sequence divergences among our 23 haplotypes ranged from 0.11% to 1.85% and the divergence of *B. bufo* from Latvian Republic (AY325988) is differ from others with a range from 0.34% to 1.6%. Populations from Turkey were closely related to those from Europe and Latvia. For 936 nucleotides, haplotypes connected by $\leqslant 17$ substitutions have at least a 0.95 probability of being parsimoniously connected and a network was generated using TCS (Clement et al., 2000) thus all the haplotypes could be joined in a single network (fig. 2).

Discussion

Partial sequences of 16S rRNA have been widely used in the assessment of relationships within and between amphibian genera. It was reported that typically variation within this region is low within species (Harris, 2001). In Asia, Liu et al. (2000) found five variable sites in geographically widely distributed popula-