Genetic confirmation of the occurrence and notes on the ecology of the yellow-bellied toad, *Bombina variegata* (L., 1758) (Amphibia: Bombinatoridae) in the European part of Turkey

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ABSTRACT - We confirmed the occurrence and report some ecological traits of the yellow-bellied toad, *Bombina variegata* from Turkish Thrace. The specimens were caught in April 2016 from ponds and several streams of Karacahasan Mountain, Enez District, Edirne Province. In order to determine and confirm its systematic position, we genetically analysed the tissue samples of caught specimens. We also present some behavioural data of the species.

INTRODUCTION

The yellow-bellied toad, *Bombina variegata* has a wide distribution in Europe and is represented by three subspecies: nominotypic subspecies *B. v. variegata* in Western Europe and Carpathian Mountains, *B. v. pachypus* in Apennine Mountains, *B. v. scabra* in Balkans (Vukov et al., 2006; Fijarczyk et al., 2011). *B. v. pachypus* is suggested as full species by Canestrelli et al., (2006). *B. v. kolombatovici* is found identical to the nominal subspecies, which is supported by mtDNA analyses (Fromhage et al., 2004).

Recently, a first record of *B. variegata* was reported from Turkey; it was affiliated to *B. v. scabra*, which is the geographically nearest subspecies and well known from the Balkans (Bülbül et al., 2016). However, the very first record of the species in Turkey was given by Boulenger (1897) as *Bombinator pachypus* (actually a subspecies of *B. variegata*) from Adrianople (ancient Roman name of Edirne Province, Turkey) without the exact location, and then mentioned by Atatür & Yılmaz (1986) who considered Boulenger's record as irrelevant. In the Bülbül et al. (2016)'s paper these two aforementioned references were ignored.

In April 2016, during a herpetological trip to the European part of Turkey, we encountered a yellow-bellied toad population in Karacahasan Mountain, Enez District, Edirne Province, which was morphologically assigned to *Bombina variegata scabra* (Küster, 1843) by Bülbül et al. (2016). The main aim of this study was confirmation of the occurrence, to genetically determine the systematic position of the found *Bombina variegata* population, and to reveal some ecological traits of the species in Turkey.

MATERIALS AND METHODS

We observed about 50 specimens (adults and juveniles) of which 19 adults were captured (9 males, 10 females). Adult males were recognised by the presence of nuptial pads on the fore limbs. We photographed all caught specimens individually and took tissue samples from the toes of all individuals. We stored the tissue samples in 96% ethanol. We afterwards released all specimens to their natural habitat.

The authors had received special permission for the fieldwork from the Republic of Turkey, Ministry of Forestry and Water Affairs, Directorate of Nature Conservation and National Parks (permit number: 2014-51946). Also, ethical permission for tissue samples from Ege University Animal Experiments Ethics Committee (permit number: 2014-002) was received.

DNA was isolated from five samples using the Qiagen Blood and Tissue Kit following the manufacturer's instructions. We sequenced a ca. 500 bp fraction of the 16SrRNA gene [primers 16SAL and 16SBH of Palumbi et al. (2002); initial melting for 120 s at 94 °C, 33 cycles of denaturation for 30 s at 94 °C, primer annealing for 30 s at 51 °C, extension for 60 s at 65 °C, final step at 65 °C for 10 min]. PCR reactions were prepared using the 5Prime Master Mix. PCR products were purified using the High Pure PCR Product Purification Kit of Roche. Sanger reactions were run using the Big Dye Terminator (ABI) with initial melting for 60 s at 96 °C, 25 cycles of denaturation for 10 s at 96 °C, primer annealing for 5 s at 50 °C, extension for 240 s at 60 °C. Single stranded fragments from both directions were sequenced on an ABI 3500 Genetic Analyzer Series 2 automatic sequencer using standard protocols.

Phylogenetic analyses were done with Mega (version 7, Kumar et al., 2016). We added homologous reference sequences from complete mitochondrial genomes of known *B. variegata* and *B. bombina* lineages (GenBank accession numbers JX893172, JX893174, JX893176, JX893177, JX893178, JX893179, EU115993 and AY971143; Pabijan et al. 2008, 2013) as well as from two Alytes species (GenBank accession numbers AY333709 and AY333710; Fromhage et al., 2004) as outgroups. Sequences were aligned with ClustalW (Thompson et al., 1994). Our

intention was not to produce a robust phylogeny of selected *B. variegata* haplotypes, rather we wanted to just barcode the new samples. We therefore only performed a reduced phylogenetic analysis. The best fitting out of 24 substitution models were selected with Mega 7 (Kumar et al., 2016) based on AICc values. For phylogenetic reconstruction we used Maximum-Likelihood (ML), with the selected substitution model (GTR+G) and 2,000 bootstrap replicates.

RESULTS AND DISCUSSION

Morphological comparison of live specimens from our study and previously published results (Radojičić et al., 2002; Vukov et al., 2006; Bülbül et al., 2016) do not differ much, so we did not present any measurements or colouration-pattern characteristics.

We observed about 50 specimens in almost all ponds and streams of the north-western parts of Karacahasan Mt., i.e., the south-eastern of Gala Lake (Fig. 1-a, b). The first record was given from one pond (181 m asl) by Bülbül et al. (2016), we restated the altitude range of the species as 85-185 m asl on Karacahasan Mountain. Specimens were found in the pond at the highest elevation (185 m asl; 40º 45' N, 26º 13' E) and on the stream side at the lowest elevation (85 m asl; 40º 45' N, 26º 13' E). The exact coordinates (second parts of the GPS) are not given for conservation purposes. Air temperature was 19 °C, while the water was colder at range between 4-7 °C. During the photography session of the individuals, some specimens showed defensive postures (unken reflex) (Fig. 1-c, d). One specimen (Fig. 1-d) did the ventral unken reflex behaviour spontaneously while we tried to catch the specimen from the pond. We moved the specimen from the water to rock surface for better photography. Also some pairs of specimens were on inguinal amplexus behaviour while they were stored together in containers before photography session and tissue sampling. Nuptial pads of the male specimens were also very distinct, so we can indicate the mating season starts from April. Habitat and behavioural photographs are given in Fig. 1.

The sympatric herptiles, *Emys orbicularis* (Linnaeus, 1758), *Lissotriton vulgaris* (Linnaeus, 1758), *Natrix natrix* (Linnaeus, 1758), *Podarcis tauricus* (Pallas, 1814), *Pelophylax ridibundus* (Pallas, 1771), *Bufotes variabilis* (Pallas, 1769) were observed.

All samples showed the same haplotype (GenBank accession numbers KY496588-KY496592). According to the results of the phylogenetic analysis, our *Bombina variegata* haplotypes and *B. v. scabra* from Bulgaria (B3 in Pabijan et al., 2013), which is geographically closest to the Turkish samples, form a clade with 93% bootstrap support [Fig. 2; topological discordance to the tree shown by Pabijan et al. (2013) is due to the reduced data set]. According to their molecular dating this lineage must have diverged from the second Balkan lineage of *B. variegata* during the Early Pleistocene.

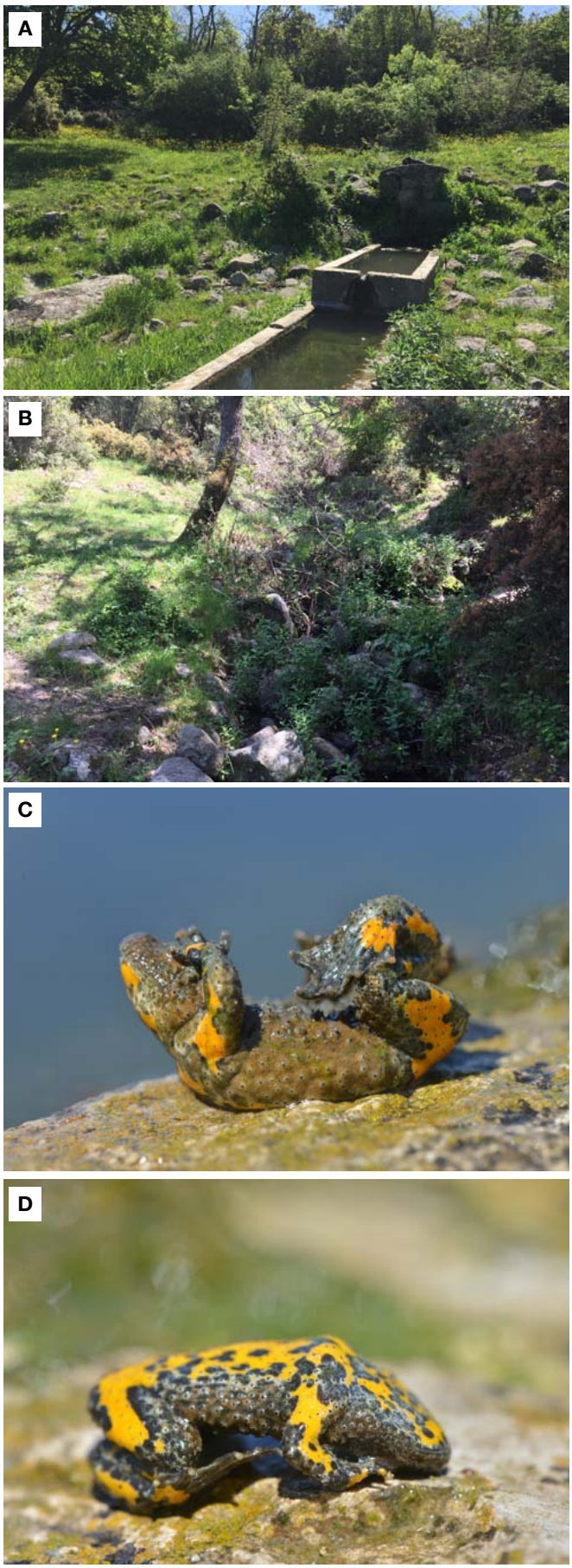

Figure 1. Pond habitat (A), stream habitat (B), dorsal unken reflex [boating] (C) and ventral unken reflex (D) of the yellow-bellied toad, *B. variegata*

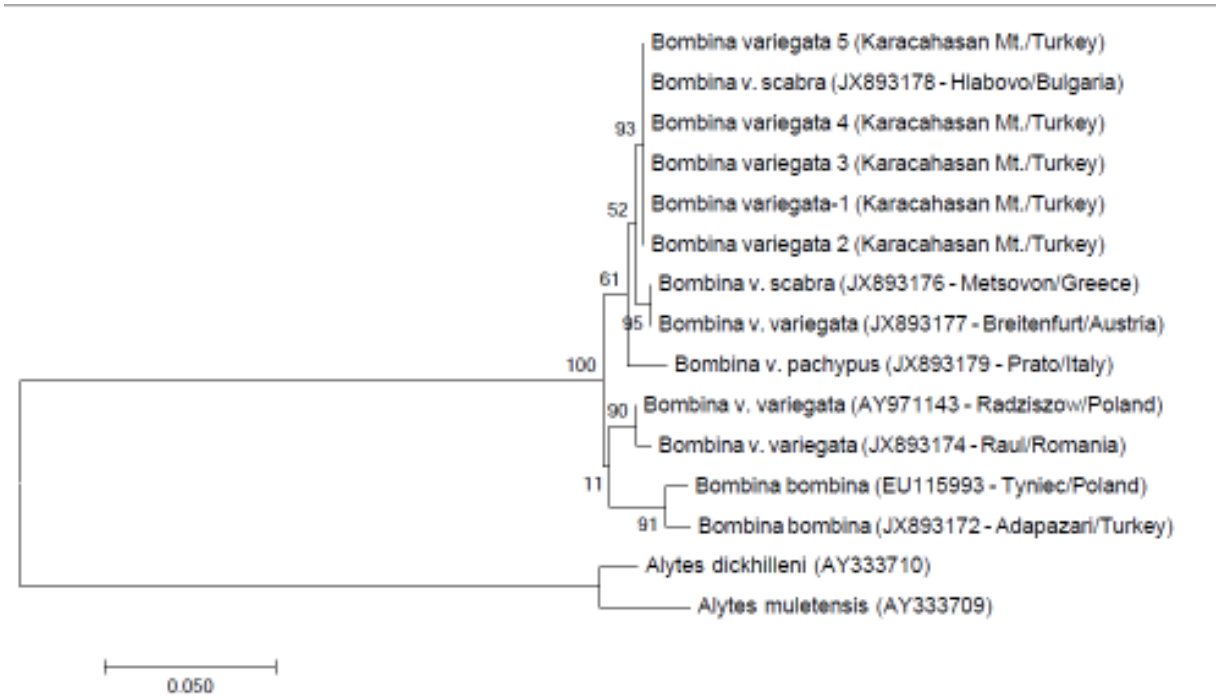

Figure 2. Maximum likelihood tree based on the GTR+G substitution model; numbers at nodes indicate bootstrap values for 2,000 replicates

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