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Biochemical Differentiation between *Bombina bombina bombina* (Linneaus, 1761) and *B. b. arifiyensis* (Ozeti and Yilmaz, 1987) as Revealed by the Skeletal Muscle Protein Bands

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Abstract: In this study, skeletal muscle protein bands of the *Bombina bombina* specimens from five populations inhabiting in Turkish Thrace and Northwestern Anatolia were compared by SDS-PAGE (Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis). Totally 54 adult (26 females and 28 males) *B. bombina* specimens were used in SDS-PAGE characterization. *B. b. bombina* specimens in the Thrace group (Degirmenyeni, Buyukdoluk and Durusu populations) had 26 homologous protein bands whereas *B. b. arifiyensis* specimens in the Northwestern Anatolia group (Arifiye and Karasu) had 28 bands. It was shown that specimens of *B. b. arifiyensis* had two additional and unique bands. The results of the present study show that Thrace and Northwestern Anatolia specimens of the *B. bombina* populations in Turkey are different according to total number of skeletal muscle protein bands.

Key words: Skeletal muscle, *Bombina bombina*, Thrace, Western Anatolia, protein band, Turkey

INTRODUCTION

The fire-bellied toad, *Bombina bombina* (Linnaeus, 1758) shows wide distribution in Eastern, Northern and Central Europe. It is critically endangered in most of its current distribution range and protected by the so-called habitat directive of the European Union (Stuckas and Tiedemann, 2006). It is also found in Northwest Anatolia. *B. bombina* is the only representative of the Bombinatoridae living in Turkey where it is confined to small areas in Turkish Thrace and Northwestern Anatolia (Alpagut-Keskin *et al*., 2010). Both Thracian and Anatolian populations of *B. bombina* are located as peripheral isolates on the Southeast of the Southern margin of the species' range (Mayr, 1963). Anatolian populations of *B. bombina* which are isolated geographically by the Sea of Marmara have been described as a subspecies, *B. bombina arifiyensis* (Ozeti and Yilmaz, 1987) by Yilmaz (1984, 1986), Ozeti and Yilmaz (1987) and Baran and Atatur (1998) according to morphological investigations. Additionally, Ozeti and Arikan (1989) reported that *B. b. arifiyensis* specimens in Adapazari were different from *B. b. bombina* specimens in Edirne according to their some globulin (G1 and G4) fractions. Arikan *et al*. (2010) adressed a different perspective and they compared the size and counts of various blood cells of *B. bombina* specimens belonged to Anatolian and Thracian populations. Nevertheless, the magnitude and pattern of genetic differentiation of Anatolian *B. bombina* from the European and Thracian populations have not been shown (Alpagut-Keskin *et al*., 2010). The European fire-bellied toad species have broad geographical distributions with mutually exclusive ranges. In *B. bombina*, two closely related groups were delineated by allozyme variants: a Northern one, inhabiting lowlands North of the Carpathian Mountains and a Southern one, distributed along the Danubian plains (Szymura *et al*., 2000; Stuckas and Tiedemann, 2006; Voros *et al*., 2006; Hofman *et al*., 2007). Szymura *et al*. (2000) which also included an Anatolian sample of this species, attempted to analyze mtDNA variation in fire bellied toads but did not include the Thracian populations. The sample from Anatolia although, geographically distant was found to be more similar to the Northern group than the Southern *B. bombin*a (Szymura *et al*., 2000). Alpagut-Keskin *et al*. (2010) used information from 20 allozyme loci to test the hypothesis that geographically isolated Anatolian populations of fire-bellied toad showed genetic differentiation when compared with Thracian populations located on the other side of the Bosporus. They reported that the extent and patterns of genetic divergence indicated that the Anatolian and Thracian populations had probably experienced bottlenecks and incipient speciation might have occurred in Anatolian populations of *B. bombina*. To extend these data showing morphological, serological and allozyme differences

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among the Thracian and Anatolian fire-bellied toads in Turkey by a different perspective, the purpose of the present study is to perform a biochemical comparison on the skeletal muscle proteins of the *B. b. bombina* specimens from three populations (Buyukdolluk, Degirmenyeni and Durusu) with *B. b. arifiyensis* ones from two populations (Arifiye and Karasu) in Turkey for the first time by SDS-PAGE.

MATERIALS AND METHODS

Total of 54 adult (26 females and 34 males) *B. bombina* specimens were captured from five populations in Turkey (Table 1) and sampling areas were shown in the Fig. 1. LatLon coordinates of the populations were shown in the Table 2. In each SDS-PAGE experiment, one specimen was used for each population and the experiments were repeated for all specimens in each population. Skeletal muscle protein samples of the frogs obtained by grinding 0.1 g skeletal muscles of each specimens in liquid nitrogen and by adding 0.1 mL of DDW (Double Distilled Water) and 0.2 mL of 2× SDS gel-loading buffer (100 mM Tris-Base pH 6.8, 4% SDS electrophoresis grade, 0.2% bromophenol blue and 20% glycerol) (Sambrook *et al*., 1989). The samples were boiled for 2 min in the 2×SDS gel-loading buffer to denature the proteins prior to loading the samples onto gels (Lutz *et al*., 2001). SDS-PAGE 99 programme was used for boiling in a thermal block. The size of the minigels was 8.3×7.3 cm and the resolving gels were 12% (w/v) gradient. The 12% gradient gels were prepared by putting 3.3 mL of DDW, 4 mL of 30% acrylamide mix, (29.2% acrylamide and 0.8% N, N'-methylene-bis-acrylamide) 2.5 mL of 1.5 M Tris pH 8.8, 0.1 mL of 10% SDS, 0.1 mL of 10% ammonium persulfate and 0.004 mL of TEMED into a beaker. The 5% stacking gels were prepared by putting 2.7 mL of DDW, 0.67 mL of 30% acrylamide mix, 0.5 mL of 1.5 M Tris pH 6.8, 0.04 mL

Table 1: Locality names and voucher numbers of the studied specimens

Subspecies name	Locality	n	Voucher number
B. b. bombina	Edirne (Buyukdolluk)	10 (6♂, 4♀)	TrUfB-01-10
B. b. bombina	Edirne (Degirmenyeni)	10 (5♂, 5♀)	TrUfB-11-20
B. b. bombina	Istanbul (Durusu)	11 (6♂, 5♀)	TrUfB-21-31
B. b. arifiyensis	Sakarya (Karasu)	11 (5♂, 6♀)	TrUfB-32-42
B. b. arifiyensis	Sakarya (Arifiye)	12 (6♂, 6♀)	TrUfB-43-54

Table 2: Names and LatLon coordinates of the studied populations.

		n		
Number	Population name	♂♂	♀♀	LatLon coordinates
1	Edirne (Buyukdolluk)	6	4	41°45'50"N 26°35'16"E
2	Edirne (Degirmenyeni)	5	5	41°46'22"N 26°33'81"E
3	Istanbul (Durusu)	6	5	41°19'06"N 28°40'52"E
4	Sakarya (Karasu)	5	6	41°05'57"N 30°39'13"E
5	Sakarya (Arifiye)	6	6	40°42'52"N 30°21'44"E

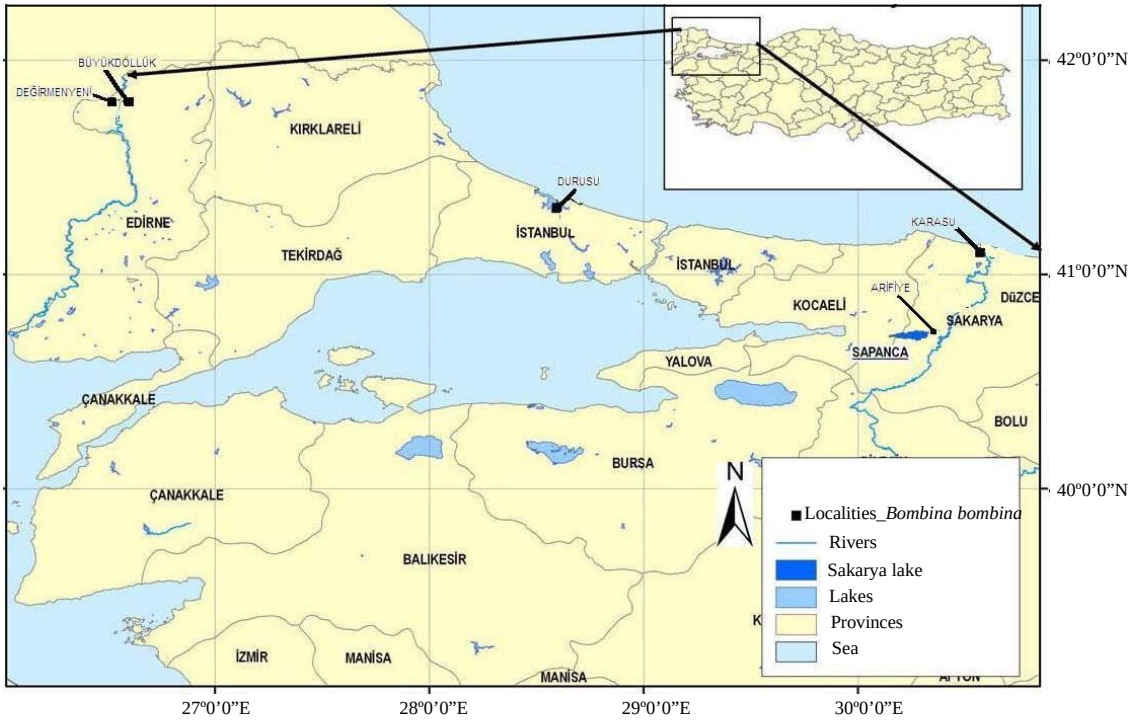

Fig. 1: Map showing the localities of the studied samples

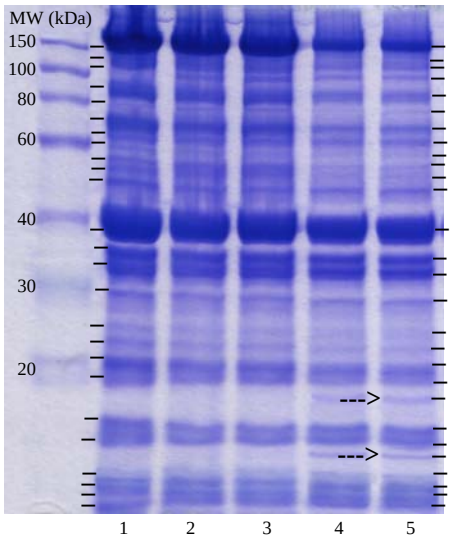

Fig. 2: SDS-PAGE comparison of the five different *Bombina bombina* specimens from Black sea Region. About 20 μL (36 μg) of protein samples were applied to the wells. Bars were added upon the best visible protein bands. The two additional and unique bands belonged to the Northwestern Anatolian specimens were shown by the arrow. Numbers from 1-5 represent the names of the populations as followings. 1: Degirmenyeni, 2: Buyukdolluk, 3: Durusu, 4: Arifiye and 5: Karasu

of 10% SDS, 0.04 mL of ammonium persulfate and 0.004 mL of TEMED into a beaker (Sambrook *et al*., 1989). For SDS-PAGE experiments, 1.80 μg protein of each 1 μL sample was applied to the wells. SERVA recombinant SDS-PAGE protein marker (10-150 KDa Plus, Liquid mix) was used to estimate the positions of the protein bands. Gels were run at a constant current of 20 mA for 60 min (Lutz *et al*., 2001).

Coomassie Brillant Blue (CBB) R-250 was used to stain the gels. The gels were put into the washing solution 1 which was prepared by mixing 50 mL of methanol, 10 mL of glacial acetic acid and 40 mL of DDW for 60 min. Then, the gels were taken to washing solution 2 prepared by 7 mL of glacial acetic acid, 5 mL of methanol and 88 mL of DDW for 60 min. Finally, the gels were scanned in a scanner and they were shown in Fig. 2.

RESULTS AND DISCUSSION

Skeletal muscle protein bands of three different *B. b. bombina* specimens from Thrace and two different *B. b. arifiyensis* specimens from Northwestern Anatolia populations were compared. Total 26 homologous protein bands were found in Thrace group (Degirmenyeni, Buyukdolluk and Durusu populations) while the Northwestern Anatolia group (Arifiye and Karasu) had 28 bands. The additional 2 protein bands were shown in Fig. 2. Some of the vague bands were not taken into account.

All specimens were used in the SDS-PAGE experiments and it was not found any differences between males and females. The number of skeletal muscle protein bands was always 26 for Thrace group and 28 for Northwestern Anatolia group when researchers used all specimens in different gels. Only the best visible gel is shown in Fig. 2. All these different gels had the same vague bands.

SDS-PAGE results showed that total number of skeletal muscle protein bands was 26 in Thracian group while there were 28 homologous bands in Northwest Anatolian group. Using the total number skeletal muscle protein bands to compare the different amphibian populations was also performed by Bulbul and Kutrup (2007, 2011) and Kutrup and Bulbul (2011).

The results of the study performed by Bulbul and Kutrup (2007) on the total number of protein bands of the green toad (*Bufo viridis*) showed that Hatay specimens were similar to Kayseri, Rize and Tekirdag specimens while Mersin specimens had 2 different protein bands compared to other specimens in their study.

Bulbul and Kutrup (2011) indicated that the number of skeletal muscle protein bands different in two distinct Turkish marsh frog species (*Pelophylax ridibundus* and *Pelophylax caralitanus*). On the other hand, Kutrup and Bulbul (2011) stated that number of skeletal muscle protein bands also could be a good tool to show the biochemical differences between different subspecies.

Based on their SDS-PAGE characterization, the number of the skeletal muscle protein bands was found different between the populations belonged to two different subspecies, *Ommatotriton ophryticus ophryticus* and *O. o. nesterovi* of the Northern banded newt, *Ommatotriton ophryticus*. Similar to these studies on amphibians, Hasnain *et al*. (2005) studied the soluble muscle proteins in four fish species.

The researchers found that 16 protein bands were diagnostic to *Channa gachua* and *Channa striatus* while 10 and 15 bands were to *C. marulus* and *C. punctatus* by SDS-PAGE. These data revealed that total number of

skeletal muscle protein bands and molecular weights of skeletal muscle proteins could be different either among species or subspecies. The results showed that skeletal muscle protein bands were different between Thracian and Northwestern Anatolian samples by SDS-PAGE. Conformably with the SDS-PAGE comparison, Alpagut-Keskin *et al*. (2010) analyzed 20 allozyme loci from Thracian and Northwestern Anatolian's *B. bombina* specimens by polyacrylamide gel electrophoresis. They reported that Thracian and Northwestern Anatolian specimens were different.

In parallel, Ozeti and Arikan (1989) reported that *B. b. arifiyensis* specimens in Adapazari were different from *B. b. bombina* specimens in Edirne according to globulin fractions. Arikan *et al*. (2010) used a different approach and they indicated that Northwestern Anatolian *B. bombina* samples share general features with other anurans but differ from other populations of the same species in blood cell parameters.

In line with electrophoretic studies, Yilmaz (1984, 1986), Ozeti and Yilmaz (1987) and Baran and Atatur (1998) stated that Anatolian populations of *B. bombina* have been described as a subspecies, *B. bombina arifiyensis* according to morphological investigations.

CONCLUSION

In this study, differences in the number of skeletal muscle protein bands of the Thracian *B. b. bombina* samples from three populations (Degirmenyeni, Buyukdolluk and Durusu) and Northwestern Anatolian *B. b. arifiyensis* specimens from two populations (Arifiye and Karasu) are consistent with the previous morphological and biochemical data.

The study provides additional data on biochemical knowledge of the *Bombina bombina* specimens in Turkey. Based on the the protein SDS-PAGE results, researchers suggest to follow the common idea proposing that *Bombina bombina* is subdivided into two geographic fragments: the Thracian group is allocated to the *B. b. bombina* subspecies while Northwestern group is named as *B. b. arifiyensis*.

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