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Biochemical Differentiation Between *Hyla orientalis* (Bedriaga, 1890) and *Hyla savignyi* (Audoin, 1827) as Revealed by the Skeletal Muscle Protein Bands

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Abstract: In this study, in order to investigate biochemical differences among skeletal muscle protein bands of the *Hyla orientalis* specimens from three populations and *H. savignyi* specimens from six populations in Turkey were compared by SDS-PAGE (Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis). Totally, 26 adult (14 females and 12 males) *H. orientalis* specimens and 54 adult (25 females and 29 males) *H. savignyi* specimens were used in SDS-PAGE characterization. The first group (Bursa, Edirne and Trabzon populations of *H. orientalis*) had 34 homologous protein bands whereas the second group (Adana, Hatay, Gaziantep, Kilis, Siirt and Elazig populations of *H. savignyi*) had 32 bands. It was shown that the first group had two additional and unique bands. The results of the present study show that specimens of the *H. orientalis* and *H. savignyi* populations in Turkey are biochemically different according to total number of skeletal muscle protein bands.

Key words: Skeletal muscle, SDS-PAGE, tree frogs, group, Turkey

INTRODUCTION

The tree frog, *Hyla arborea* (Linneaus, 1758) is widely distributed throughout most of Europe (except Southern France, Iberia and Balearic Islands) and Asia Minor (Mertens and Wermuth, 1960; Terentiev and Chernov, 1965). Previously, it was recognized that there were three subspecies of *Hyla arborea*: *Hyla arborea arborea* (Bird, 1936; Bodenheimer, 1944; Nikolsky, 1962; Lambert, 1970; Clark and Clark, 1973), *Hyla arborea savignyi* (Bird, 1936; Schmidt, 1939; Bodenheimer, 1944; Nikolsky, 1962; Clark and Clark, 1973) and *Hyla arborea kretensis* (Wettstein, 1953). After Schneider and Nevo (1972), the tree frogs inhabiting the southern part of Turkey were accepted as a distinct species, *Hyla savignyi* in several studies (Engelman *et al*., 1985; Leviton *et al*., 1992; Winden and Bogaerts, 1992; Bohme and Wiedl, 1994). Based on colour pattern (Duellman, 1977) and bioacoustic properties (Schneider and Nevo, 1972; Schneider, 1974; Kaya and Simmons, 1999) substantial differences between *H. savignyi* and *H. arborea* were reported. Kaya and Simmons (1999) stated that both species, *Hyla arborea* and *Hyla savignyi* were present in Turkey according to their bioacoustical data and they found a remarkable correlation between the external morphology and the advertisement calls in the populations studied. Kaya (2001) also reported that there were two different species, *Hyla arborea* and *Hyla savignyi* in Turkey according to morphological data. Gvozdik *et al.* (2008) reported that *Hyla arborea* and *Hyla savignyi* are closely-related species distributed in Europe and the Middle East. They supported that the geographically close but ecologically vicariant populations of *H. arborea* and *H. savignyi* species from the Caucasus region differ quite substantially in body shape. On the other hand, Stock *et al.* (2008) stated that Southeastern European and Western Anatolian specimens of *H. arborea* should be considered a separate species, *Hyla orientalis* (Bedriaga, 1890). *H. orientalis* is distributed parapatrically Northward and westward from the range of *H. savignyi* in Asia Minor and presumably in the Caucasus (Gvozdik *et al*., 2010; Gvozdik, 2010). Gvozdik *et al.* (2010) reported that two different tree frog species *Hyla orientalis* and *Hyla savignyi* inhabit Turkey according to mtDNA, rhodopsin and tyrosinase data. Gul *et al.* (2012) supported the existing of two distinct tree frog species from Turkey based on mitochondrial and nuclear DNA data. On the other hand, they reported that *H. savignyi* species comprised of two sublineages and detailed investigations between these sublineages were needed. Finally, using ecological niche modeling Gul (2013) examined ecological information to define ecological divergence between two lineages of *Hyla savignyi* and he stated that separate analysis of the lineages showed no overlap of their predicted ranges based on climatic data of their respective habitats.

To extend these data showing morphological, mtDNA and allozyme differences among the Turkish tree frogs by

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a different perspective, the purpose of the present study is to compare skeletal muscle proteins of the *H. orientalis* specimens from three populations with *H. savigyni* ones from six populations in Turkey for the first time by SDS-PAGE.

MATERIALS AND METHODS

Totally, 26 adult (14 females and 12 males) *H. orientalis* specimens were captured from three populations (Bursa, Edirne and Trabzon) and totally 54 adult (25 females and 29 males) *H. savignyi* specimens were captured from six populations (Adana, Hatay, Gaziantep, Kilis, Siirt and Elazig) in Turkey (Table 1). LatLon coordinates of the studied populations were given in the Table 1. Sampling localities were shown in the Fig. 1. 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 were 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 X 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 X 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 per sulfate 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 of 10% SDS, 0.04 mL of ammonium per sulfate 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 shown in Fig. 2.

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

Species name	Locality	n	LatLon coordinates	V. number
H. orientalis	Bursa (Orhangazi)	4♂, 5♀	40°30'04"N29°18'38"E	TrUAI-01-09
H. orientalis	Trabzon (Of)	5♂, 5♀	40°56'42"N40°16'08"E	TrUAI-10-19
H. orientalis	Edirne (Buyukdolluk)	3♂, 4♀	41°45'35"N26°36'13"E	TrUAI-20-26
H. savignyi	Adana (Seyhan)	5♂, 4♀	36°55'23"N35°10'09"E	TrUAI-27-35
H. savignyi	Hatay (Antakya)	5♂, 4♀	36°12'00"N36°10'34"E	TrUAI-36-44
H. savignyi	Gaziantep (Merkez)	4♂, 3♀	37°03'55"N37°23'00"E	TrUAI-45-51
H. savignyi	Kilis (Sunnep Golu)	5♂, 5♀	36°48'04"N37°07'26"E	TrUAI-52-61
H. savignyi	Siirt (Ziyaret Beldesi)	5♂, 4♀	38°07'02"N41°43'30"E	TrUAI-62-70
H. savignyi	Elazig (Taþoren Koyu)	5♂, 5♀	38°42'45"N39°46'13"E	TrUAI-71-80

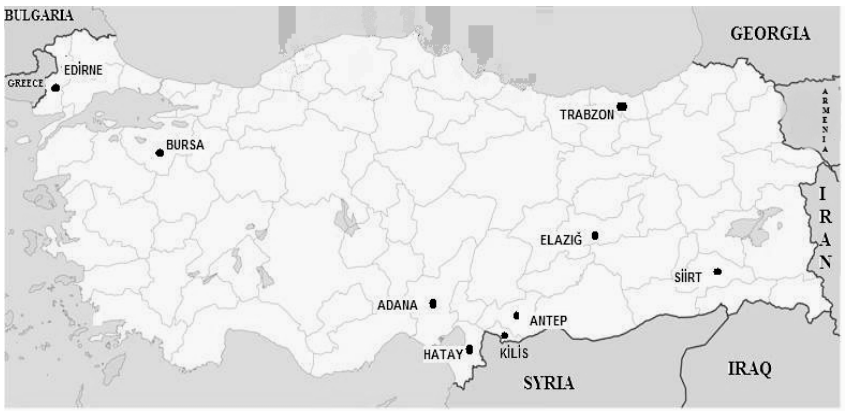

Fig. 1: Map showing the localities of the studied samples

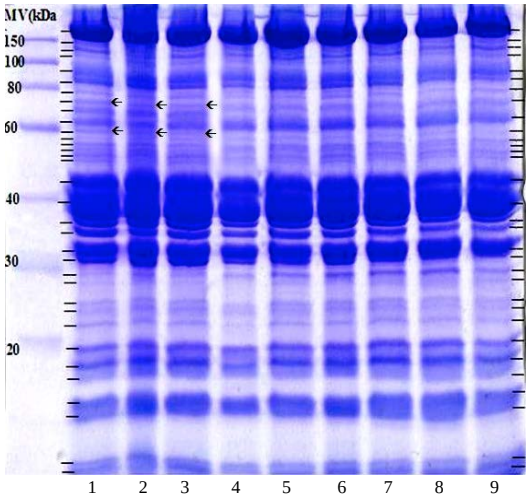

Fig. 2: Comparison of skeletal muscle protein bands between *Hyla orientalis* and *Hyla savignyi* specimens from Turkey. The 20 μL (36 μg) of protein samples were applied to the wells. Bars were added upon the best visible protein bands. The two additional and unique bands belonged to the *Hyla orientalis* specimens were shown by the arrow. Numbers from 1-9 represent the names of the populations as followings. 1: Bursa; 2: Trabzon; 3: Edirne; 4: Adana; 5: Hatay; 6: Gaziantep; 7: Kilis; 8: Siirt and 9: Elazig

RESULTS

Skeletal muscle protein bands of *H. orientalis* and *H. savignyi* specimens were compared. Totally, 34 homologous protein bands were found in first group (Edirne, Bursa and Trabzon populations of *H. orientalis*) while the second group (Elazig, Siirt, Kilis, Gaziantep, Hatay and Adana populations of *H. savignyi*) had 32 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 no differences were found between males and females. The number of skeletal muscle protein bands was always 34 for the first group and 32 for the second group when we used all specimens in different gels. Only the best visible gel is given in Fig. 2. All these different gels had the same vague bands.

DISCUSSION

In this study, we compared skeletal muscle protein bands of the *H. orientalis* samples from three populations and *H. savignyi* samples from six populations in Turkey by SDS-PAGE.

SDS-PAGE results showed that the total number of skeletal muscle protein bands was 34 in *H. orientalis* samples while there were 32 bands in *H. savignyi* samples. Using the total number skeletal muscle protein bands to compare the different animal populations has been subjected to the recent studies. Hasnain *et al.* (2005) studied the soluble muscle proteins in four fish species. 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. Bulbul and Kutrup (2007) used SDS-PAGE characterization to compare different populations of the green toad (*Bufo viridis*) in Turkey. The results on the total number of protein bands of the green toad showed that the specimens of one population had 2 different protein bands compared to other specimens in their study. Another study on taxonomical status of Turkish marsh frogs was conducted by Bulbul and Kutrup (2011). They stated that the total number of skeletal muscle protein bands was 27 in *Pelophylax ridibundus* specimens whereas *Pelophylax caralitanus* specimens had 28 bands. 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*. 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. As it can be seen from the results of the present study, differences in the number of skeletal muscle protein bands of the *H. orientalis* and *H. savigyni* specimens are consistent with the previous morphological and molecular data. Although, recent literature clearly revealed the genetic differences between two distinct tree frog species in Turkey, Gul *et al.* (2012) stated that *H. savigyni* specimens formed two different sublineages (sublineage1 from Adana, Mersin, Osmaniye, Kayseri and Igdir; sublineage 2 from Hatay, Kahramanmaras, Kilis, Gaziantep, Sanliurfa, Mardin and Elazig) based on mitochondrial DNA data. Gul *et al.* (2012) addressed the necessity of comprehensive investigations on the sublineages of *H. savignyi* from Southeastern Turkey. Finally, Gul (2013) used Ecological Niche Modeling Methods and he stated that the Anatolian Diagonal clearly forms a genetic and geographic barrier between lineages of *H. savignyi*. In addition, this barrier reveals different microhabitat preferences of the lineages. He

mentioned that detailed morphological observations might reveal evidence for a possible subspecies of *H. savignyi* in Turkey. But he clearly stated that his hypothesis needs further study with inclusion of morphological data. Contrary to findings of Gul *et al.* (2012) and Gul (2013), when we compared skeletal muscle protein bands of Adana (sublineage1) specimens with Hatay, Gaziantep, Kilis and Elazig (sublineage2) ones, we could not observed any differences between the sublineages of *H. savignyi*. The study comparing skeletal muscle protein bands not only provides additional data on biochemical knowledge of the *H. orientalis* and *H. savignyi* specimens but also shows skeletal muscle protein similarity between sublineages of *H. savignyi* in Turkey.

CONCLUSION

In this study, differences in the number of skeletal muscle protein bands of *H. orientalis* samples from three different populations and *H. savignyi* samples from six different populations are consistent with the previous morphological and biochemical data. The study provides additional data on biochemical knowledge of tree frogs in Turkey. Based on the protein SDS-PAGE results, we conclude that there is no difference between two sublineages of *H. savignyi*.

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