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Comparative morphology of urohyal bone in brackish water species of the genus *Aphanius* Nardo, 1827 in the Persian Gulf and Southeastern Mediterranean Sea basins (Teleostei: Aphaniidae)

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Abstract

Among the skeletal elements in fishes, the urohyal bone which lies in the lower part of the head - the central part of the mandibular skeleton- has proved to be of special significance in fish systematics. In the present study, the urohyal bones of six brackish water *Aphanius* species (i.e., *Aphanius hormuzensis*, *A. stoliczkanus*, *A. furcatus*, *A. ginaonis*, *A. mento*, *A. sirhani*) were compared using morphological description and linear measurements to explore the effectiveness of this structure in the separation of the studied *Aphanius* species. The description of the urohyal bones and their morphological variations allowed identification of *A. furcatus*, *A. mento* and *A. sirhani* from their relatives. Moreover, the urohyal height/urohyal length (UH.UL) significantly separated *A. hormuzensis* from *A. ginaonis*, and the maximum height/urohyal length (MH.UL) significantly discriminated *A. mento* from its relatives. Discriminant function analysis (DFA) separated the studied species with high classification success (overall mean 94.7%). These results showed the power of urohyal bone morphology in separating the *Aphanius* species analyzed, and highlighted the taxonomic value of the urohyal bone. The observed variation in the urohyal bone of *Aphanius* species is largely consistent with their phylogeny. This designated that at least some morphological features of the urohyal bone in *Aphanius* are probably encoded by genetic factors, which can be further used for species discrimination.

Keywords: Hard structures; Morphology; Aphaniidae; Taxonomy; Multivariate analysis.

Introduction

The urohyal bone is a single bony structure located in the ventral part of the head in fishes and formed as ossification of an unpaired tendon of the sternohyoideus muscle (Arratia & Schultze, 1990). Its anterior tip is generally connected to the ventral hypohyal, the antero-dorsal part to the first basibranchial and the posterior part is connected to the shoulder girdle by a large muscle (Kusaka, 1974) (Fig. 1). Among the osteological elements of the teleostean fish, the urohyal bone has proved to be of exceptional significance in fish systematics (Yazdani & Prakash, 1990).

The urohyal attachment to the hypohyal is commonly thickened and sometimes forked to allow the connections of a paired ligament to the ventral hypohyals (Yazdani & Prakash, 1990). Therefore, this bone plays an important role in the mouth opening-closing mechanism of a fish.

The most extensive work on urohyal bone has been done by Kusaka (1974) in which different groups of fishes were classified according to the variations in their urohyal bone morphology. He demonstrated a relationship

between the fish morphology and its biological behaviors with the shape of their urohyal bone. He eventually concluded that the fishes with slender heads have elongated urohyals, the high-bodied fishes have vertically expanded urohyals, the active swimmer fishes have spatula-like shaped urohyals with an undeveloped ventral spread, and the omnivorous fishes have urohyals with a developed ventral spread.

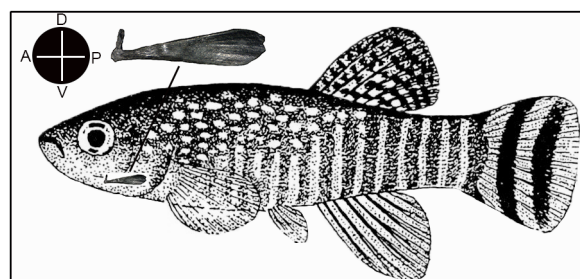


Fig. 1: Position of the urohyal bone in the oral cavity of an *Aphanius*. The photo is *A. ginaonis* from Wildekamp (1993).

The urohyal bone is considered as synapomorphy for the members of teleost fishes (De Pinna, 1996). Further studies indicated that morphological features of the urohyal bone can be used to discriminate fish families, genera and even species (Arratia & Schultze, 1990; Jawad *et al.*, 2016), and that urohyal bone has taxonomic significance (Kusaka, 1974; Esmaeili & Teimori, 2006). Therefore, it can be hypothesized that urohyal bone contains sufficient data to disclose taxonomic and even phylogenetic relationships among the fishes.

However, and despite the diagnostic values of the urohyal bone in teleost fishes, its morphological characteristics have not been studied sufficiently for many fishes. The qualitative and quantitative studies of the urohyal bone would be particularly interesting for the discrimination of those fish taxa that are similar in their external morphology (Manizadeh, 2017). A good example is the members of the killifish, genus *Aphanius* Nardo 1827, where most of the species are morphologically similar, and difficult to discriminate.

The genus *Aphanius* (commonly known as toothcarps in the studied regions) is the only representative of the cyprinodontid fishes in the Mediterranean Sea basin, the Red Sea, Persian Gulf basins, as well the inland waters of Turkey and Iran (Wildekamp, 1993; Teimori, 2013). Even though the members of the genus *Aphanius* can be separated by using molecular markers in term of phylogeny, it is difficult to distinguish them by considering only fish external morphology even in multivariate spaces (Teimori *et al.*, 2012a). Therefore, it is necessary to accomplish comparative analyses and investigate morphological characteristics of the hard structures such as scales (e.g. Gholami *et al.*, 2013; Teimori *et al.*, 2017a-

b), otolith (e.g. Reichenbacher *et al.*, 2009a-b; Teimori *et al.*, 2012a-c) and urohyal bone because their differences might be indicative of taxonomic value or phylogenetic signal.

This study presents for the first time an attempt to describe morphological characteristics of the urohyal bone in six brackish water species of the genus *Aphanius* and to assess the possible contribution of this hard structure in their discrimination.

Materials and Methods

Studied species and dissection of the urohyal bone

The materials in this study include four species from the Persian Gulf region (Hormuzgan and Mond basins) and two species from the Southeastern Mediterranean Sea basin. Based on a very recent study by Teimori *et al.* (2018), the *Aphanius* populations in Hormuzgan basin (Southern Iran) are defined as new distinct species, *Aphanius hormuzensis* Teimori, Esmaeili, Hamidan, Reichenbacher, 2018 and those from the Mond basin are revised as *Aphanius stoliczkanus* (Day, 1872). The materials from the Persian Gulf region consist of; *Aphanius hormuzensis*; *A. furcatus* Teimori, Esmaeili, Erpenbeck, Reichenbacher, 2014; *A. ginaonis* (Holly, 1929) (Hormuzgan basin); and *Aphanius stoliczkanus* (Day, 1872) (Mond basin). The materials from Southeastern Mediterranean Sea basin include; *A. mento* Heckel, 1843 and *A. sirhani* Villwock, Scholl & Krupp, 1983 (Table 1 and Fig. 2).

Specimens were chosen without separating the sexes because of the low number of individuals in each individual sexe. The fish standard length (SL) ranges from

Table 1. Overview of the studied *Aphanius* species, number of individuals (N), sampling sites, habitat types and drainage basins where specimens have been collected. SD = standard deviation.

Species	N	Site	Habitat type	Basin	Country	SL (mean ± SD)
<i>A. hormuzensis</i>	4	Mehran 1	Brackish water river	Hormuzgan, Persian Gulf	Iran	
	3	Mehran 2	Brackish water river	Hormuzgan, Persian Gulf	Iran	
	4	Kol	Brackish water river	Hormuzgan, Persian Gulf	Iran	30.6 ± 4.40
	3	Rassul	Brackish water river	Hormuzgan, Persian Gulf	Iran	
	4	Khurgu	Hot sulphuric spring	Hormuzgan, Persian Gulf	Iran	
<i>A. stoliczkanus</i>	10	Howba	Hot sulphuric spring	Mond, Persian Gulf	Iran	32.0 ± 2.00
<i>A. furcatus</i>	4	Faryab	Hot sulphuric spring	Hormuzgan, Persian Gulf	Iran	25.3 ± 1.50
	4	Kol	Brackish water river	Hormuzgan, Persian Gulf	Iran	
<i>A. ginaonis</i>	10	Genow	Hot sulphuric spring	Hormuzgan, Persian Gulf	Iran	31.2 ± 1.35
<i>A. mento</i>	8	Beirut	Brackish water river	Southeastern Mediterranean Sea	Leobnan	28.7 ± 1.12
<i>A. sirhani</i>	8	Jordan	Brackish water river	Dead Sea, Southeastern Mediterranean Sea	Jordan	27.8 ± 1.10

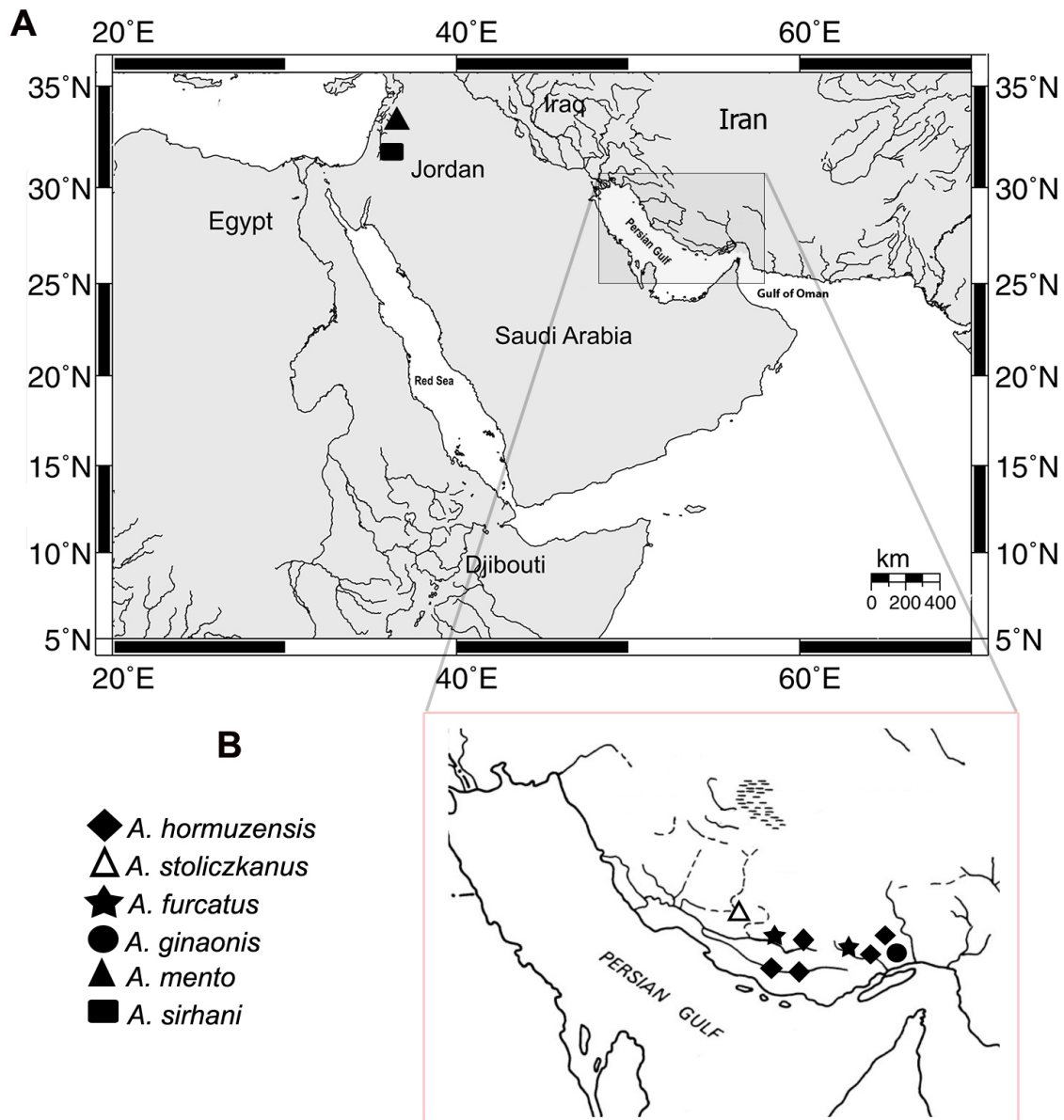


Fig. 2: Overview of the location of the studied *Aphanius* species in Persian Gulf region (below, the black symbols are from Hormuzgan basin, and the white triangular symbol is from Mond basin) and Southeastern Mediterranean Sea drainages (above). Map source: Wildekamp (1993).

23 to 42 mm. A total of 62 urohyal bones were extracted from all the species studied. The flesh was removed by placing the alcohol preserved (96% ethanol) fish specimen in boiling water for five minutes and the urohyal bone was extracted from the ventral side of the head, washed and stored dry in small envelopes. All of the extracted urohyal bones are catalogued and deposited in the Zoological Museum of Shahid Bahonar University of Kerman (ZM-SBUK). Digital image of each urohyal bone was taken from left side using a digital camera (model SP-320®, Olympus) connected to stereo-microscope (model SZ61® Olympus, Tokyo, Japan).

Urohyal bone description and measurement

To describe the urohyal bone morphology, we followed the terminology used in Chollet-Villalpando *et al.* (2014a) (Fig. 3). Accordingly, the urohyal shape was defined as follows; urohyal bone short (urohyal length (UL) < urohyal height (UH)), broad (urohyal height > 1/2 urohyal length); long (urohyal length > urohyal height) or narrow (urohyal height < 1/2 urohyal length). Also, some other characters such as urohyal dorsal length (DL), urohyal maximum height (MH), urohyal height (UH), urohyal length (UL), and ventral length (VL) were measured

following Kusaka (1974) (Fig. 4A). All the measurements were carried out using ImageJ 1.x (Schneider *et al.*, 2012). To remove the effect of fish size on the data, all of the measurements were standardized as a function of the urohyal length (Lahnsteiner & Jagsch, 2005). Therefore, the values of selected measured morphometric parameters (=Mmp) were standardized by using the urohyal length (i.e. Mmp/UL*100). Eventually, the relative features were calculated for qualitative analysis (Table 2). In addition, three angles including condylar angle (CA), ventral angle (VA) and posterodorsal angle (PDA) were measured and compared among the studied species (Fig. 4B).

Analysis of data

Because of the vulnerability of some studied species such as *Aphanius ginaonis* and *A. sirhani*, we used low sample size (an average of around 10 per species) in our study. The Kolmogorov-Smirnov (K-S) test of normality at the 95% confidence level was used to test normal distribution of the data. It showed that our data are normally distributed ($P > 0.05$). Discriminant function analysis (DFA) as a multivariate analysis was used to show the possible discrimination of the studied species based on the relative characters of their urohyal bones. One-way

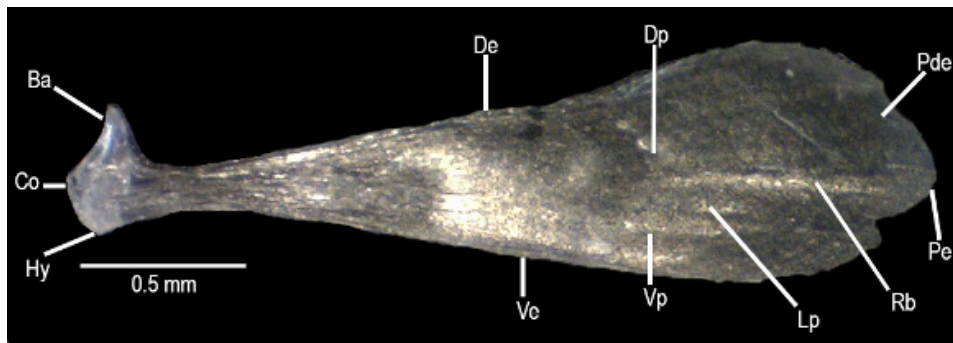


Fig. 3: Left side view of the urohyal bone of *Aphanius* species and its terminology. Ha, hypohyal attachment; Ba, basibranchial attachment; Ve ventral extension; De dorsal extension; Dp dorsal plate; Pde posterodorsal edge; Rb radial band; Lp lateral plate; Pe posterior edge; Ve ventral edge; Vp ventral plate; Co condyle (adapted from Kusaka, 1974).

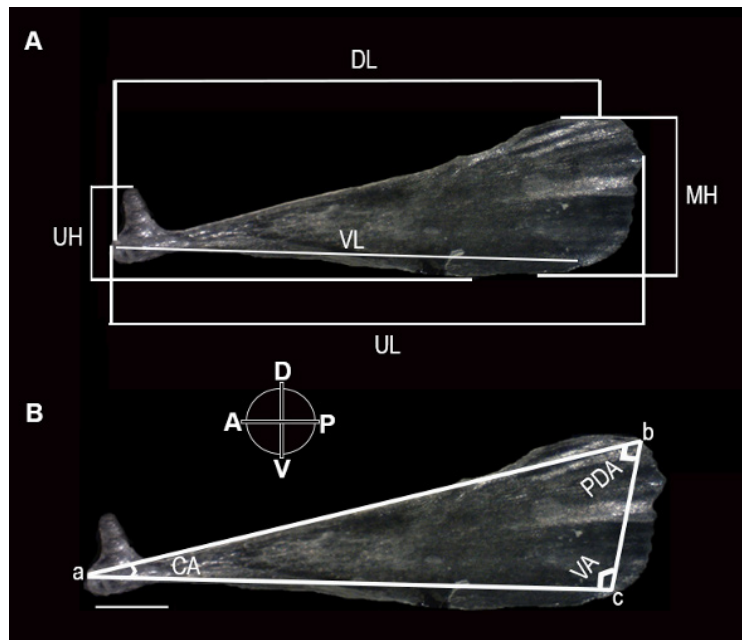


Fig. 4: Linear measurements (A) and three measured angles (B) of the urohyal bone of an *Aphanius* species based on Kusaka, 1974. The measured parameters are as follow: DL, dorsal length; MH, maximum height; UH urohyal height; UL urohyal length; VL ventral length; CA condylar angle (angle 1; BAC); PDA posterodorsal angle (angle 2; ABC); VA Ventral angle (angle 3; ACB). The urohyal photo is from a left side view.

Table 2. Values of the urohyal variables (means and standard deviations) among the studied *Aphanius* species (one-way ANOVA with Duncan post-hoc test, $P < 0.05$); N, number of individuals. Angle 1 is the Condylar angle (CA in Fig. 2b), Angle 2 is the posterodorsal angle (PDA in Fig. 2b) and angle 3 is the ventral angle (VA in Fig. 2b). SD = standard deviation.

Variable	<i>A. hormuzensis</i> N=18	<i>A. stoliczkanus</i> N=10	<i>A. furcatus</i> N=8	<i>A. ginaonis</i> N=10	<i>A. mento</i> N=8	<i>A. sirhani</i> N=8
	Mean ±SD (min-max)	Mean ±SD (min-max)	Mean ±SD (min-max)	Mean ±SD (min-max)	Mean ±SD (min-max)	Mean ±SD (min-max)
Angle1	77.8±11.5 (58.4-109.0)	65.9±10.2 (58.5-73.5)	79.4±5.2 (74.3-84.9)	77.4±1.2 (71.2-80.7)	76.4±3.1 (67.5-79.1)	72.8±2.5 (66.7-76.7)
Angle2	83.3±12.2 (52.9-103.9)	96.5±10.5 (89.1-103.8)	83.3±6.5 (79.1-90.8)	80.8±6.4 (76.2-85.3)	74.1±1.2 (73.2-75.0)	85.5±2.7 (83.7-87.5)
Angle3	144.1±16.5 (109.7-168.3)	148.2±17.2 (135.6-160.7)	139.5±20.2 (120.6-160.8)	91.3±5.7 (87.2-95.3)	91.2±0.3 (91.3-91.4)	74.4±1.3 (73.5-75.3)
DL.UL	81.9±7.5 (65.8-92.8)	90.2±3.7 (87.5-92.7)	80.2±10.5 (68.8-89.6)	86.1±3.7 (83.3-88.8)	90.5±10.6 (88.2-91.6)	96.8±0.3 (96.6-97.0)
VL.UL	83.4±6.1 (66.1-89.2)	86.7±1.4 (85.4-87.7)	91.4±4.5 (86.2-94.4)	94.0±2.2 (92.4-95.6)	90.2±2.5 (88.2-92.1)	82.3±0.5 (81.9-82.7)
UH.UL ^a	18.9±5.5 (11.4-29.6)	28.4±1.7 (27.1-29.6)	28.2±2.6 (25.5-30.9)	13.1±3.1 (10.9-15.3)	21.9±7.9 (16.3-27.6)	26.4±1.4 (25.4-27.4)
MH.UL ^b	27.5±3.5 (22.3-35.2)	25.4±4.4 (22.3-28.6)	28.3±3.2 (25.9-30.9)	29.6±5.1 (26.1-33.2)	34.2±1.0 (33.7-34.3)	31.9±2.1 (30.4-33.4)

a: separates significantly *A. hormuzensis* and *A. ginaonis* from its compared relatives

b: separates significantly *A. mento* from its compared relatives

ANOVA with Duncan post-hoc test ($P < 0.05$) as a univariate analysis was used to test if significant differences exist among the studied species with regard to their urohyal bones. In addition, a dendrogram was created by hierarchical cluster analysis (Unweighted Pair Group Method with Arithmetic Mean, UPGMA), based on the Euclidian distance of the urohyal bone variables to assess the degree of phenetic similarity between the studied *Aphanius* species. All of the statistical analyses were carried out in IBM (SPSS ver. 24).

Results

General characteristics of the urohyal bones in *Aphanius* spp.

General morphology of the urohyal bones in the studied species is illustrated in Figures 5–6. The urohyal bone length (UL) in all of these species is higher than the urohyal height (UH), which implies that urohyal bone is elongated in all of the studied species, while its morphology is variable among the species. Also, three angles, condylar angle (Angle 1, CA in Fig. 4B), posterodorsal angle (Angle 2, PDA in Fig. 2B) and ventral angle (Angle 3, VA in Fig. 2B) show ranges of 16.50–109.04, 73.50–168.37 and 52.93–103.93 degrees, respectively. Generally, the urohyal bones in these species consist of a basibranchial (Ba), hypohyal (Hy) and condyle (Co) attached to the anterior

margin. These structures show considerable variations among the studied species with regard to their shapes. In addition, in some species an extension or process with different shapes can be seen at the anterior side of the urohyals. The condyle at the anterior side of urohyal may be short or long, thick or thin, and or compressed in the hypohyal union. The urohyals have four distinguishable edges including; dorsal (De), ventral (Ve), posterior (Pe) and posterodorsal (Pde) edges (Fig. 3). Radial band (Rb) is long or short, and dorsal plate (Dp) is usually wide. Ventral edge is mostly straight, lateral plate (Lp) is mostly wide, and ventral plate (Vp) is often narrow.

Description of urohyal bone for the Persian Gulf brackish water *Aphanius* species

Aphanius hormuzensis (Fig. 5a-g). Description of the urohyal bone for the *A. hormuzensis* is based on 18 specimens collected from five sites in the Hormuzgan basin, Southern Iran (Table 1 and Fig. 2). The urohyal in this species is long ($UL=3.27 > UH=0.48$), basibranchial attachment at its anterior margin is either moderately elongated and thickened (Fig. 5a-e, g) or thin (Fig. 5f); the hypohyal attachment is often thickened at its anterior margin, and moderately extends in some habitats (Fig. 5a-d); condyle is short and thick and compressed in the hypohyal union; dorsal edge is often smooth and elevated to the dorsal extension; radial band is often long (Fig.

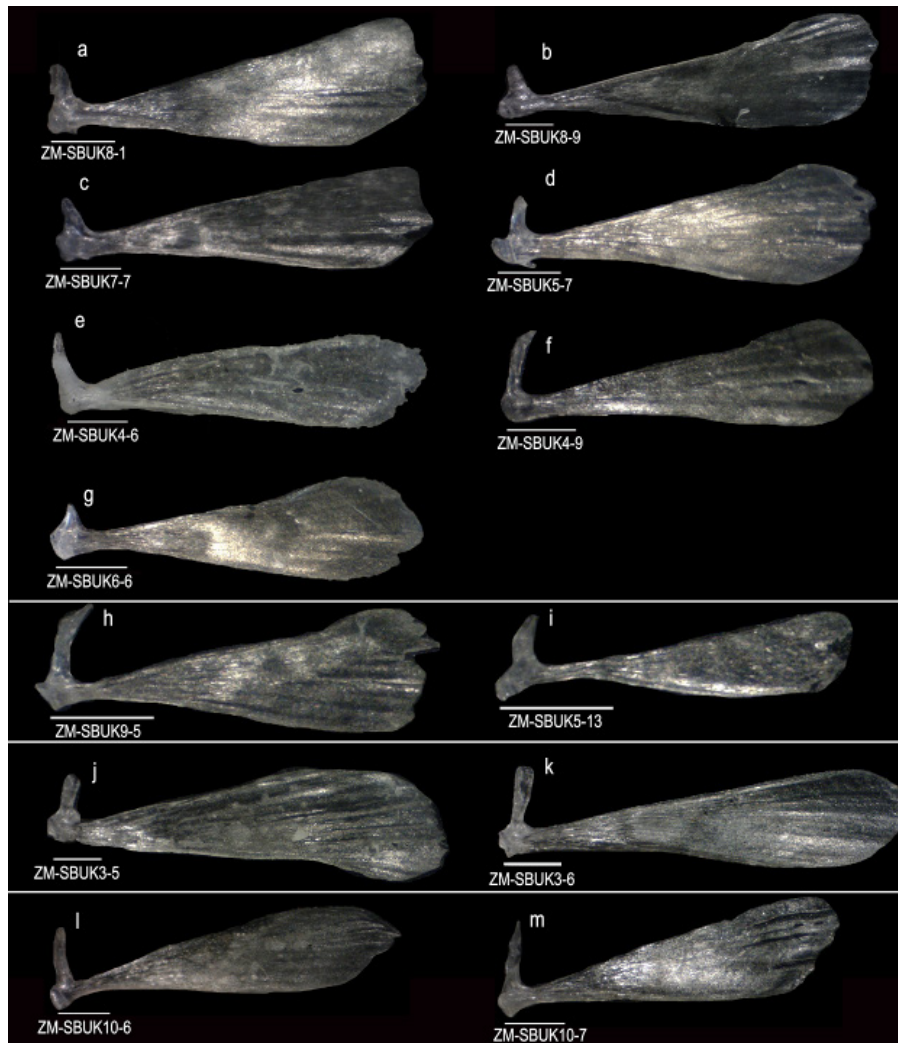


Fig. 5: Lateral view of the urohyal bone of the studied *Aphanius* species from the Persian Gulf region with their corresponding voucher numbers indicated below the urohyal bones. a-g *Aphanius hormuzensis*; h-l *A. furcatus*; j-k *A. ginaonis*; i-m *A. stoliczkanus*. Scale bar 0.5 mm.

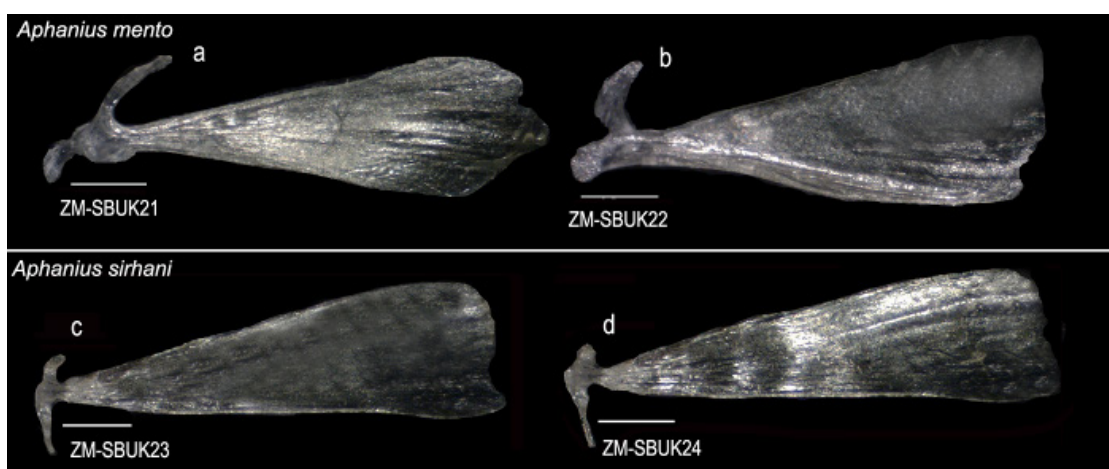


Fig. 6: Lateral view of the urohyal of the studied *Aphanius* species from Southeastern Mediterranean Sea basin with their corresponding voucher numbers indicated below the urohyal bones. a-b *Aphanius mento*; c-d *A. sirhani*. Scale bar 0.5 mm.

5a-f) with an extension to posterior edge. The posterodorsal edge is mostly longer than the ventral and dorsal edges with an exception observed in Figure 5a-b.

Aphanius furcatus (Fig. 5 h-i). Description of the urohyal bone for the *A. furcatus* is based on eight specimens collected from two habitats in the Hormuzgan basin, Southern Iran (Table 1 and Fig. 2). The urohyal bone from Faryab hot sulphuric spring was coded as ZM-SBUK9-5, and that from Kol brackish water River was coded as ZM-SBUK5-13 (Fig. 2). The urohyal bone in *A. furcatus* is long ($UL=1.71>UH=0.48$), basibranchial attachment is prominently elongated and thin in the urohyals of both habitats (Fig. 5h-i). The hypohyal attachment is thickened at its anterior margin in the specimen from Faryab hot sulphuric spring (Fig. 5h) although it is moderately thin and extends in the specimens from Kol River (Fig. 5i); condyle is short and uniform in the ZM-SBUK9-5 (Fig. 5h), while it is not developed in the ZM-SBUK5-13 (Fig. 5i); the dorsal edge is often smooth and elevated to the dorsal extension; radial band long with an extension to the posterior edge. The posterior edge is neither equal (Fig. 5h) nor shorter (Fig. 5i) than the posterodorsal and ventral edges.

Aphanius ginaonis (Fig. 5 j-k). Description of the urohyal bone for the *A. ginaonis* is based on the ten specimens collected from Genow hot sulphuric spring in the Hormuzgan basin, Southern Iran (Table 1 and Fig. 2). The urohyal bone in this species is long ($UL=2.76>UH=0.30$); basibranchial attachment is elongated and thin; hypohyal attachment is not developed at its anterior margin; condyle is short and uniform; dorsal edge is often smooth and elevated to the dorsal extension; radial band is long with an extension to the posterior edge. The posterior edge is almost longer than the posterodorsal and ventral edges (Fig. 5j-k).

Aphanius stoliczkanus (Fig. 5 l-m). Description of the urohyal bone for the *A. stoliczkanus* is based on the ten specimens collected from Howba hot sulphuric spring, the Mond basin, Southern Iran (Table 1 and Fig. 2). The urohyal bone in this taxon is long ($UL=3.04>UH=0.86$); basibranchial attachment is strongly elongated and thin; the hypohyal attachment is often thickened at its anterior margin, and moderately extended; condyle is short and uniform; the dorsal edge is often smooth and elevated to the dorsal extension; radial band is short with an extension to the posterior edge. The posterior edge is almost longer than the posterodorsal and ventral edges (Fig. 5l-m).

Description of urohyal bone for Southeastern Mediterranean Sea brackish water *Aphanius* species

Aphanius mento (Fig. 6 a-b). Description of the urohyal bone for the *Aphanius mento* is based on the eight specimens collected from Jordan River drainage basin (Table 1 and Fig. 2). In *A. mento* urohyal bone is long ($UL=4.0>UH=0.93$); basibranchial attachment is strongly elongated and thin, and is bent toward the posterior region; the hypohyal attachment is thickened at its anterior

margin, and moderately extended; condyle is long and extended; the dorsal edge is smooth and elevated to the dorsal extension; radial band is long with an extension to the posterior edge. The posterior edge is either longer than the posterodorsal and ventral edges (Fig. 6a) or equal to them (Fig. 6b).

Aphanius sirhani (Fig. 6 c-d). Description of the urohyal bone for the *Aphanius sirhani* is based on the eight specimens collected from Dead Sea basin (Table 1 and Fig. 1). In this species, urohyal is long ($UL=3.26>UH=0.83$); the basibranchial attachment is almost short and thickened, and bent toward the posterior region (Fig. 6c); the hypohyal attachment is strongly elongated and thin at its anterior margin; condyle is short and uniform; the dorsal edge is smooth and elevated to the dorsal extension; radial band is short with an extension to the posterior edge. The posterior edge is shorter than the posterodorsal and ventral edges (Fig. 6c-d).

Morphometric analyses

The results of the descriptive analysis based on the four standardized urohyal variables and the three angles are summarized in Table 2. The univariate analysis showed that the two (out of four) morphometric characters, including; $UH.UL$, $MH.UL$, and the condylar angle (CV in Fig. 4B), differ significantly among the studied species (ANOVA with post hoc Duncan test, $p<0.05$). The urohyal high/urohyal length ($UH.UL$) significantly separates *A. hormuzensis* and *A. ginaonis* from all the other studied species (Table 2). Moreover, the urohyal maximum height/urohyal length ($MH.UL$) significantly discriminates *A. mento* from all the other studied relatives (ANOVA with post hoc Duncan test, $p<0.05$).

Discriminant Function Analysis (DFA), based on all the morphometric variables and the three angles, separates the studied species with high overall classification success (94.7%) (Table 3). Additionally, the average linkage dendrogram based on the Euclidean distance was calculated for all the morphometric variables and angles. The resulted dendrogram categorizes the studied species into two major clusters, in which cluster I includes *A. hormuzensis*, *A. stoliczkanus* and *A. furcatus*, and cluster II contains *A. ginaonis*, *A. mento*, and *A. sirhani* (Fig. 7). Within the cluster I, *A. hormuzensis* is grouped to the *A. stoliczkanus*, and these together clusters with *A. furcatus*. Within the cluster II, *A. mento* is grouped to the *A. sirhani* and these together cluster with *A. ginaonis*.

Discussion

The urohyal bone is one of the hard structures located in the central part of mandibular skeleton of teleost fishes (Kusaka, 1974). It plays an important role not only in mouth opening-closing mechanism but also in the trophic strategies of fishes (Arratia, G., Schultze, 1990; Chollet-Villalpando *et al.*, 2014b). Considering its function, the urohyal bone can be used in feeding studies to quan-

Table 3. Classification success of the DFA (jack-knifed) based on all four standardized variables and three angles of the urohyals of studied *Aphanius* species. Overall classification success is 94.7% (Wilks' $\lambda=0.05$ for function 1 and 0.36 for function 2). N, number of individuals.

species	Predicted Group Membership						N
	<i>A. hormuzensis</i>	<i>A. stoliczkanus</i>	<i>A. furcatus</i>	<i>A. ginaonis</i>	<i>A. mento</i>	<i>A. sirhani</i>	
<i>A. hormuzensis</i>	90.0		10.0				18
<i>A. stoliczkanus</i>		100.0					10
<i>A. furcatus</i>			100.0				8
<i>A. ginaonis</i>				100.0			10
<i>A. mento</i>					100.0		8
<i>A. sirhani</i>						100.0	8

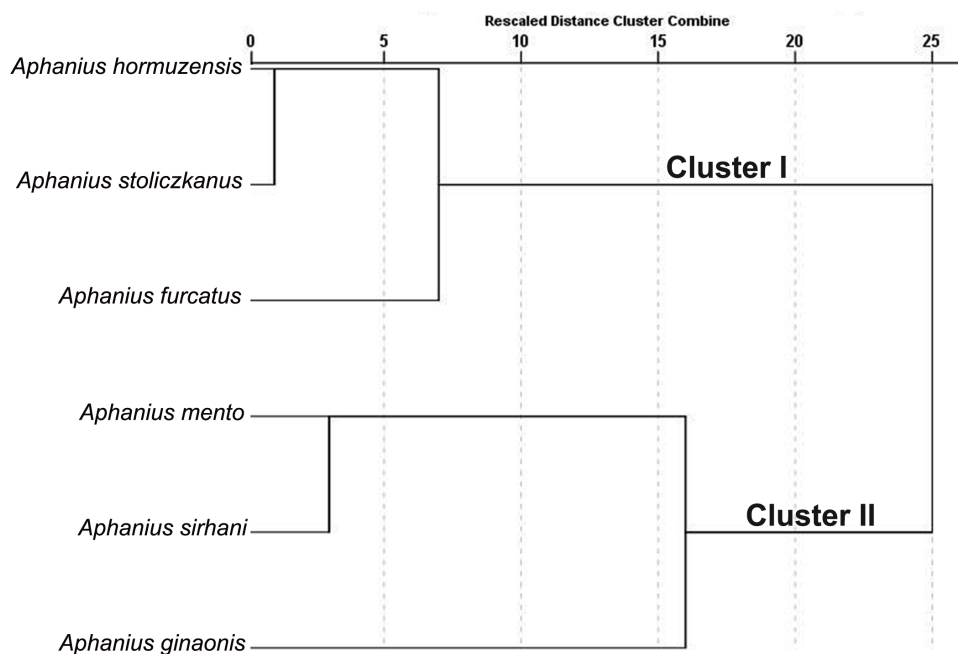


Fig. 7: Phenogram based on the morphometric variables indicating the phenotypic relationship of the urohyal bone morphology for the studied *Aphanius* species. Dissimilarity based on the Euclidian distance of Mahalanobis distance values, grouped by hierarchical cluster analysis (UPGMA).

tify the food consumed by piscivorous fish and also other aquatic animals (Johal et al., 2000; Tombari et al., 2010; Perez-Comesaña et al., 2013).

In addition to its biological values in fishes, the urohyal bone has also been used by ichthyologist to study fish taxonomy (Kusaka, 1974; Esmaeili & Teimori, 2006; De La Cruz-Agüero et al., 2012). Therefore, morphological characteristics of urohyal bone have long been applied for species discrimination in terms of taxonomy and phylogeny (Kusaka, 1974; Yazdani & Prakash, 1990; Murray & Attia, 2004; Mabee et al., 2011; De La Cruz-Agüero et al., 2012; Marceniuk et al., 2012; Chollet-Villalpano et al., 2014a). One of the most comprehensive studies on systematic significance of the urohyal bone has been

carried out by Kusaka (1974). After doing a comparative study on the urohyal bones of approximately 700 spp, belonging to 460 genera, 184 families, and 21 orders, he definitely concluded that urohyal morphology is obviously specific in different groups of teleost fishes. Therefore, it can be safely used for classifying families, genera, and species.

As mentioned above, most of the *Aphanius* species are similar in their external morphology, which has made them a difficult fish group to be identified for several years (Coad, 2000; Teimori et al., 2012a; Hrbek et al., 2006). Therefore, the objective of this study was to evaluate whether the urohyal bone morphology would be able to discriminate the selected *Aphanius* species. A further

aim was to see if variation seen in the urohyal bone morphology is consistent with the current classification of the studied species.

The results of this study clearly indicated that two morphological variables including urohyal height/urohyal length and urohyal maximum height/urohyal length contributed to the separation of the studied *Aphanius* species. In addition, the shapes of the basibranchial and hypophyal were characters that separated species of the Southeastern Mediterranean Sea (*A. mento* and *A. sirhani*) from their relatives in the Persian Gulf.

Even though the urohyal bones of *A. hormuzensis* from the Hormuzgan basin in southern Iran were generally characterized by moderately elongated and thickened basibranchial attachment, some intraspecific variations exist in the urohyals of this species collected from different habitats. The urohyals of *A. hormuzensis* shown in Figure 5 a-d and g are from brackish water river, while those in Figure 5e-f are from hot sulphuric spring. A clear difference can be seen in the shape of basibranchial attachment in the anterior part of its urohyals. This difference may be resulted from their different feeding habits since those specimens inhabiting in brackish water rivers feed on alga on rocks and those living in hot sulphuric springs search and feed on the soft bed of spring.

Aphanius ginaonis is another species in the Hormuzgan basin which is geographically and genetically close to the *A. hormuzensis* (Teimori *et al.*, 2014, 2018). Its urohyal bone is morphologically similar to *A. furcatus* and *A. stoliczkanus*. However, this was not supported by UPGMA analysis, in which *A. ginaonis* clustered in *A. mento* and *A. sirhani* group (Fig. 7).

The UPGMA analysis based on all the morphometric variables of the urohyal bones indicated that species of the Persian Gulf basin (with an exception of *A. ginaonis*) grouped into the same cluster. In this group, *A. hormuzensis* from Hormuzgan is clustered with *A. stoliczkanus* from the Mond basin. This is in agreement with the results of Teimori *et al.* (2012a) where they analysed fish body and otolith morphology and found a close relationship between these two species (Teimori *et al.*, 2012a; page 301, Fig. 6). Another taxon in this group is *A. furcatus* whose urohyal bone was morphologically similar to the *A. hormuzensis*. These two species are sympatric and close in term of phylogeny (Teimori *et al.*, 2014). In the same habitat, *A. hormuzensis* is usually found in the middle part of the river where water depth is higher, while *A. furcatus* tends to prefer the river sides where water is shallow. The similar morphology in their urohyal bones can be interpreted as close feeding habits of these species. However, this assumption needs further examination.

The urohyal bones of the two studied species from the Southeastern Mediterranean Sea basin were completely different from their relatives in the Persian Gulf (see also Figs.5-6). This separation based on urohyal bone morphology can also be supported by their phylogenetic relationships (Hrbek & Meyer, 2003; Teimori *et al.*, 2014; Freyhof *et al.*, 2017). *Aphanius mento* is reported

to be found in shallow water among aquatic plants and algae, where it feeds on insect larvae, crustaceans, and algae (Krupp & Schneider, 1989). *Aphanius sirhani* also lives in the same condition and is found in shallow water among aquatic plants and stones, or over muddy grounds, and feeds on insect larvae and crustaceans (Krupp & Schneider, 1989). Teimori *et al.* (2014) have found that tricuspid teeth in *A. ginaonis* and *A. furcatus* are morphologically close to those of *A. mento*, which may account for the close feeding habit of these species. It is in agreement with our finding which evidenced the morphological similarity of their urohyal bones.

According to the evidence achieved in this study, and by considering the current phylogeny of the studied *Aphanius* species (Hrbek & Meyer, 2003; Teimori *et al.*, 2014), it can be concluded that taxonomy of the genus *Aphanius* based on urohyal morphology is largely consistent with its phylogeny and that at least some morphological characters of the urohyal bone in *Aphanius* are genetically encoded. As mentioned above, urohyal bone is one of the most functional structures in fishes, and its position is related to the functional differences of the mouth-opening mechanism. Therefore, its shape differentiation might play an important role in the life history of fish and its feeding habits.

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