Combining morphology, scanning electron microscopy, and molecular phylogeny to evaluate the taxonomic power of scales in genus *Aphanius* Nardo, 1827 (Teleostei: Cyprinodontidae)

Azad Teimori, Mina Motamedi, Mahbubeh Shaker Golmakan

Abstract. Scale morphology and its structural details were studied with morphometry, geometric morphometrics, and scanning electron microscopy in five Iranian *Aphanius* species and examined comparatively with molecular phylogeny. Scales were cycloid and circular in *A. farsicus* Teimori, Esmaeili & Reichenbacher and *A. vladykovi* Coad, cycloid and polygonal in *A. isfahanensis* Hrbek, Keivany & Coad and *A. sophiae* (Heckel), and were spined and polygonal in *A. dispar* Rüppell. Univariate analysis indicated that the relative length of anterior margin of radii played a significant role in the separation of some species studied (P < 0.05). Canonical Variates Analysis (CVA) based on seven landmarks showed clear separation between the Iranian inland and inland-related *Aphanius* species (IIRAS) group (four species) and the brackish water species (*A. dispar*). The lepidonts were conical in the IIRAS group with the exception of *A. isfahanensis* (rounded) and were rounded in *A. dispar*. Based on the basal position of the brackish water *Aphanius* species in the phylogenetic trees of the genus *Aphanius,* it can be assumed that the presence of rounded lepidonts was a primary state in their scales or is the result of convergent evolution. Also, the polygonal state in the scales of *Aphanius* species was a primitive shared character state.

Keywords: Cyprinodontiformes, lepidont, phylogeny, scale topology, geometric morphometrics, Iran

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Introduction

Iran is known as speciation center for the members of the genus *Aphanius* Nardo 1827. To date, 14 species have been reported from this country, of which 12 are endemic (Hrbek et al. 2006, Coad 2009, Esmaeili et al. 2014, Gholami et al. 2014, Teimori et al. 2012a, 2014, 2016). Although these species are clearly distinguishable by genetic markers, most of them are similar in their external morphology. Therefore, in addition to the use of molecular markers in recent years (e.g., Hrbek et al. 2006, Esmaeili et al. 2014, Gholami et al. 2014, Teimori et al. 2014), other tools, including fish anatomy and hard structures, have been used for species identification (Gholami et al. 2014, Teimori et al. 2014) and even population discrimination (Gholami et al. 2013).

A few studies have already been addressed the power of the scale morphology in the classification of *Aphanius* species and also in understanding of population variation (e.g., Ferrtito et al. 2009, Gholami et al. 2013). In the current research, we used four data sets, including scale morphometric variables, geometric morphometric landmarks, scanning electron microscopy, and molecular sequences. We focused our questions on the most relevant characteristics of scale topology and scalar denticles (lepidonts) to

complete the previous studies and to garner further knowledge on the taxonomic significance of scale morphology in the genus *Aphanius*. We discuss whether variation related to the structural details of scale morphology contain taxonomic and even phylogenetic information in the Iranian *Aphanius* species.

curling, mounted dorsal-side-up on Scanning Electron Microscopy (SEM) stubs with double self-adhesive carbon stickers, and coated with an 100 Å-thick gold layer using a Polaron E 5100. Five to twelve images per scale were captured with a LEO 1430VP at 15kV. These images were used to study the scalar denticles (lepidonts).

Materials and methods

This study considers five Iranian *Aphanius* species i.e., four endemic species belong to the Iranian inland and inland-related *Aphanius* species (IIRAS, group 1 in figure 1a-c) and include *Aphanius farsicus* from the Maharlu Lake Basin, *A. isfahanensis* from the Esfahan Basin, *A. sophiae* from the Kor River Basin, and *A. vladykovi* from the Tigris Basin, and a single native species, *A. dispar*, from the Hormuzgan Basin in Southern Iran, which is a brackish water member (group 2 in figure 1e) (Esmaeili et al. 2014).
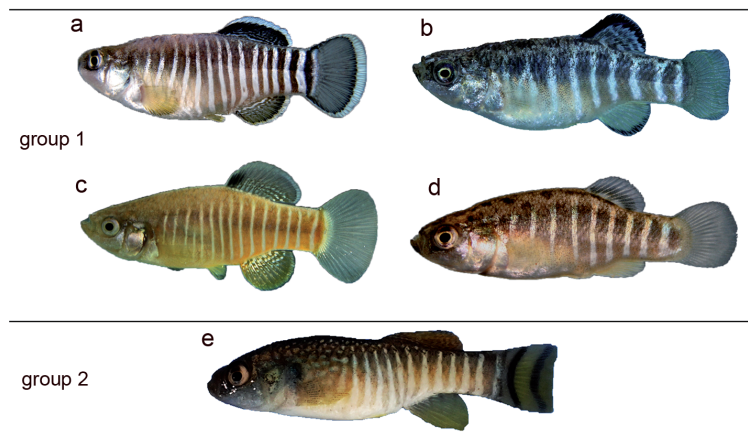

Figure 1. Photographs showing the studied Iranian inland and inland related *Aphanius* species, IIRAS (a-d) and brackish water groups (e). a to d refer to *A. farsicus*, *A. isfahanensis*, *A. sophiae* and *A. vladykovi* respectively; and e refers to *A. dispar*.

Scale extraction and examination

The fish scales from the third to fourth rows between the dorsal fin and the lateral line were removed from the left side of the specimens (Fig. 2a). These are considered to be "key scales" (Johal et el. 2006). The scales were immediately rinsed in distilled water, cleaned mechanically to remove irrelevant matter using a fine brush, and transferred into a 1% KOH solution for 40 minutes to remove soft tissues from the surface. Twenty scales per species were imaged with a digital camera connected to a Leica compound microscope taken at 15x magnification. These were used for both morphometric and geometric morphometric analyses.

The cleaned scales were dehydrated through an ascending ethanol series (30, 50, 70, and 90%) at 30 minute intervals (Lippitsch 1990), dried on Whatman filter paper, kept for several hours between two glass slides to prevent scale margin
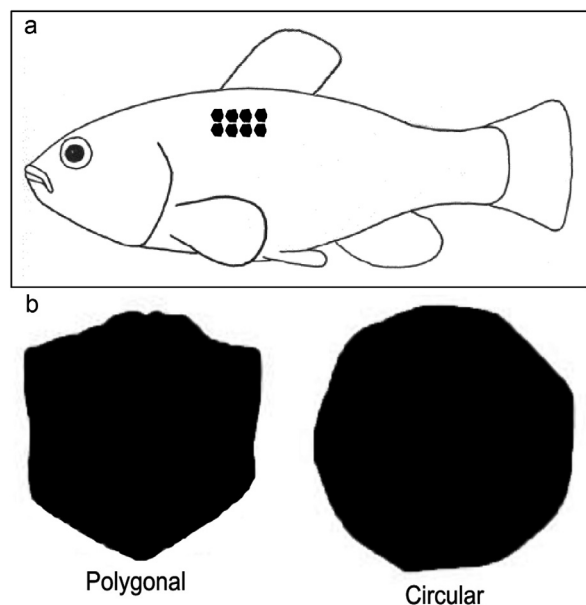

Figure 2. (a) Left side of the fish specimen shows location between the dorsal fin and lateral line, where the key scales were removed; (b) two main scale types distinguished in this study.

Table 1

Five studied linear measurements of scales. They were standardized with the following equation: ((scale character $\times$ 100)/x where, x = DVL, APL, AMR, or PMR). Finally, ten standardized variables were prepared for statistical analyses. In addition, the focal index (Fi index) was calculated as (a-f′/a-a′) $\times$ 100. See also Figure 3 for details of the characters. APL – anterior-posterior length; DVL – dorsal-ventral height; PMR – length of the posterior margin of the radii; AMR – length of the anterior margin of the radii; RL – length of the first radius

Character	Measurement	Standardized variable	Description
APL	a-a'		
DVL	b-b'	DVL.APL	(b-b'/a-a') $\times$ 100
PMR	c-c'	PMR.DVL	(c-c'/b-b') $\times$ 100
		PMR.APL	(c-c'/a-a') $\times$ 100
AMR	d-d'	AMR.DVL	(d-d'/b-b') $\times$ 100
		AMR.APL	(d-d'/a-a') $\times$ 100
		AMR.PMR	(d-d'/c-c') $\times$ 100
RL	e-e'	RL.DVL	(e-e'/b-b') $\times$ 100
		RL.APL	(e-e'/a-a') $\times$ 100
		RL.AMR	(e-e'/d-d') $\times$ 100
		RL.PMR	(e-e'/c-c') $\times$ 100
Fi index	a-f'	a-f'/a-a'	(a-f'/a-a') $\times$ 100

Scale description and morphometry, and calculating the focal index

The scale terminology follows Bräger (2016). Five linear measurements were recorded using ImageJ software (Schneider et al. 2012) as follows; anterior-posterior length (APL); dorsal-ventral height (DVL); length of the posterior margin of the radii (PMR); the length of the anterior margin of the radii (AMR); and the length of the first radius (RL) (Fig. 3). To remove the effects of allometry on scale shape, all measurements were standardized with the following equation: ((scale character $\times$ 100)/x, where, x = DVL, APL, AMR, or PMR, see Table 1). Finally, ten standardized variables were prepared for statistical analyses. The Shapiro-Wilk test showed normal distribution for the data set (P > 0.05). Therefore, univariate analysis, ANOVA with the post hoc test was used to show the significance of

differences among the species, and Canonical Discriminant Analysis (CDA) was used to show the correct classification of the studied species. The Single Linkage (nearest neighbor) method was also used to determine hierarchical clustering in our
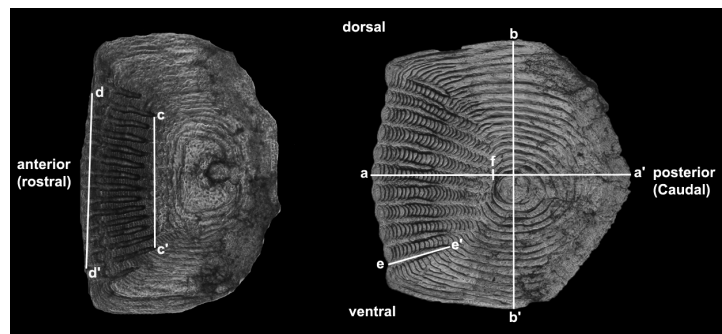

Figure 3. The linear measurements used for the morphometric analyses and also calculating of the focal index (Fi) as (a-f/a-a′)$\times$100. Anterior-posterior length (APL), dorsal-ventral height (DVL), length of posterior margin of radii (PMR), length of anterior margin of the radii (AMR), and the length of first radius (RL). "a-f" is the distance from the outermost part of the anterior field to the focus, and "a-a′" is the distance from the outermost part of the anterior field to the outermost part of the posterior field (Bräger 2016).

multivariate data sets, in which clusters were joined based on the smallest distance between two groups.

Moreover, the focal index (Fi index) was calculated to estimate the geometrical position of the focus in the studied scales as follows: (a-f/a-a′) × 100, where "a-f" was the distance from the outermost part of the anterior field to the focus and "a-a′" was the distance from the outermost part of the anterior field to the outermost part of the posterior field (Fig. 3, see Table 1). Accordingly, the position of the focus was determined as follows: $Fi < 0.20$ was the anterior position; 0.21-0.40 was the antero-central position; 0.41-0.60 was the central position; 0.61-0.80 was the postero-central position; > 0.81 was the posterior position (Bräger 2016). All analyses were performed in IBM SPSS version 22 and PAST (PAleontological Statistics, Hammer 2001).

Geometric morphometric analysis (GMA)

Here we used the same scales as before for the linear morphometric analysis. The shapes of the scale were analysed using landmark-based geometric morphometric methods (Zelditch et al. 2004). To do this, the digital images were first compiled using TpsUtil v. 1.58 (Rohlf 2015) software. According to Ibanez et al. (2007) and Requieron et al. (2012), seven landmarks were defined on each scale with tpsDig2 (Rohlf 2013) as follow (see also Fig. 4):
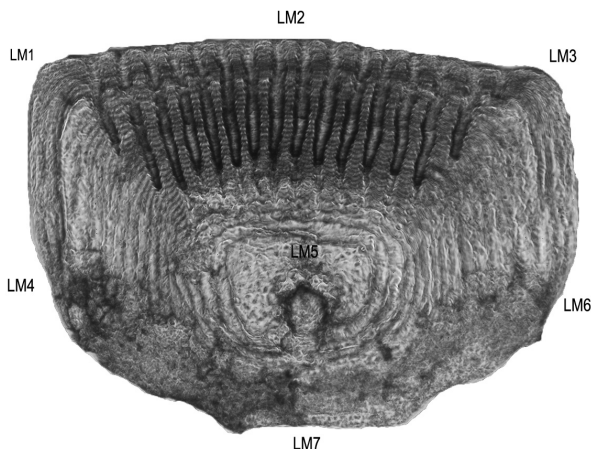

Figure 4. Landmarks used to define the shape of the scales (tooth-carp). The areas of the scales are described with respect to the fish position.

Landmark 1: the left lateral tip of the anterior portion of the scale

Landmark 2: the center of the anterior edge of the scale

Landmark 3: the right lateral tip of the anterior portion of the scale

Landmark 4: the left boundary between the anterior area with circuli and the posterior area

Landmark 5: the focus of the scale

Landmark 6: the right boundary between the anterior area with circuli and the posterior area

Landmark 7: the tip of the posterior portion of the scale.

The MorphoJ software package was used for further analysis (Klingenberg 2011). Generalized Least-squares Procrustes Superimposition (GLS) was applied to the coordinates of raw landmarks to the scales to translate and rotate them and to get new shape variables, independent of scale size (Rohlf 1990). Canonical Variates Analysis (CVA) and Discriminant Function Analysis (DFA) were performed to examine group separation. Permutation testing was performed (10000 iterations) to test the reliability of the results. To evaluate the overall pattern of morphometric relationships among the five studied species, UPGMA cluster analysis was performed on the matrix of shape distances (Euclidean Distances) using PAST (PAlaeontological Statistics, v.1.81 (Hammer et al. 2001). Moreover, Mahalanobis distance ($P < 0.0001$) was applied to show the significance of mean shape among the populations compared.

Maximum likelihood phylogenetic analysis

The maximum likelihood phylogenetic (ML) analysis is based on 15 mitochondrial cytb sequences that were deposited in Genbank by the first author (Teimori et al. 2012a, Esmaeili et al. 2014). The accession numbers of the examined sequences are as follows: *A. farsicus* (KF910701-KF910704); *A. isfahanensis* (JX154887-JX154889 and JN565969); *A. sophiae* (KJ634203-KJ634206); *A. vladykovi* (JN547802-JN547803); and *A. dispar* (JN547798).

The sequences were aligned using Muscle 3.6 (Edgar 2004), as incorporated in Seaview version 4.6.1 (Gouy et al. 2010). The ML analysis was performed using RaxML 7.2.6 (Stamatakis 2006) under the most complex GTR+G+I model of nucleotide substitution, with 2000 bootstrap replicates.

Results

Topology of the scales

The studied scales were divided into four parts: rostral and caudal fields and ventral and dorsal sides (Fig. 3). Scales imbricate each other, so only the caudal field was exposed and visible on the surface, while the rostral field lay embedded in the dermis.

Considering scale topology, two main scale types were distinguished as follows: circular (scales without additional separate ossifications) and polygonal (multi-sided forms with somehow square outline with angled corners) (Fig. 2b). In addition, the scales were classified according to the pattern on the outer edge where cycloid scales have a smooth outer edge and spined scales have spinous-like structures on the outer edge. Accordingly, scales were cycloid and circular in *A. farsicus* and *A. vladykovi*, cycloid and polygonal in *A. isfahanensis* and *A. sophiae*, and spined and polygonal in *A. dispar* (Fig. 5).

Three types of radii were present in the studied scales. Alternatively, two or all three types could be found in the scales of one species. In the rostral field of the scales, some radii failed to develop completely from the focus area (secondary radii). Therefore, the circuli remained uninterrupted in that part of the scale (originating midway between the focus and the margin). Also, some radii originated between the midway and the margin of the scale and were regarded as "tertiary radii".

Both types of radii (secondary and tertiary) are present in the scales studied in *A. farsicus* and *A. isfahanensis*. In the scales of *A. sophiae* and *A. vladykovi*, the radii were secondary and tertiary types, with secondary radii the most common type. In
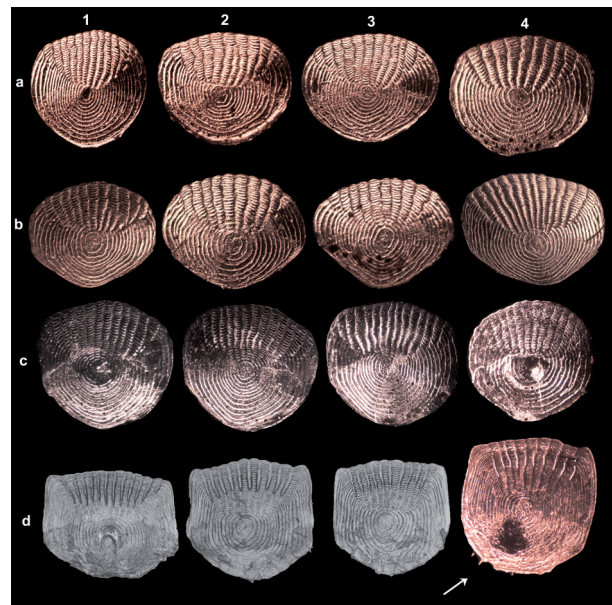

Figure 5. Photographs of light microscopy (LM) show general morphology of scales in the studied *Aphanius* species. a to d refer to *A. farsicus*, *A. isfahanensis*, *A. sophiae* and *A. vladykovi* respectively; and e refers to *A. dispar*. The arrow indicates spinous-like structures in the most posterior edge of the scales in *A. dispar*.

the rostral field of the scales in *A. dispar*, the continuity of the circuli was often interrupted by the primary radii. Secondary radii were also present, while primary radii were the most common type (Fig. 5).

As mentioned above, the position of the focus varied among the scales of the species studied. Based on the focal index, the focus in *A. farsicus* was rounded and positioned centrally (Fi = 0.46). In *A. isfahanensis* it was semicircular and positioned in the antero-central part of the scale (Fi =0.40). In *A. sophiae* it was oblong and positioned in the antero-central part of the scale (Fi = 0.35). In *A. vladykovi* it was semicircular and positioned centrally (Fi = 0.51). In *A. dispar* it was oblong and positioned centrally (Fi = 0.41).

Morphometric analyses

The results of descriptive analysis of scale variables are summarized in Table 2. Morphometric analysis was based on ten standardized scale variables. Univariate analysis shows that four of the ten morphometric characters (AMR.DVL, AMR.APL,

Table 2

Descriptive analysis (mean ± SD) of scale variables. Mean value of characters that are significantly different are indicated with an asterisk. SD – standard deviation, min – minimum, max – maximum. See also Figure 3 for details of the characters

| | Brackish water species | Inland and inland related Aphanius species (IIRAS) | | | |
| | *A. dispar* | *A. farsicus* | *A. isfahanensis* | *A. sophiae* | *A. vladykovi* |
Character	Mean ± SD (min-max)	Mean ± SD (min-max)	Mean ± SD (min-max)	Mean ± SD (min-max)	Mean ± SD (min-max)
AMR.DVL	69.7*±5.6 (60.6-76.5)	81.1±3.9 (71.7-87.3)	78.1±5.5 (70.4-93.5)	79.3±8.4 (64.5-90.5)	77.2±7.5 (72.3-84.6)
PMR.DVL	51.9±11.7 (32.2-71.1)	56.4±6.5 (40.4-78.5)	53.0±12.4 (42.4-71.1)	53.2±9.4 (44.0-66.4)	55.3±8.0 (45.0-68.6)
RL.DVL	20.1±4.1 (11.1-26.6)	15.3±3.1 (9.1-19.8)	20.8±3. 4 (10.4-26.3)	24.0*±4.9 (10.7-40.4)	17.2±9.1 (12.3-21.6)
AMR.APL	74.4*±8.1 (66.6-94.4)	96.2±6.3 (89.6-111.5)	83.6±7.4 (72.6-96.5)	82.6±8.1 (69.6-105.9)	97.2±11.5 (92.2-112.5)
PMR.APL	55.5±13.1 (33.1-77.1)	66.1±10.2 (51.5-89.8)	56.7±14.1 (45.6-76.5)	55.3±9.6 (42.1-73.1)	70.2±0.6 (55.3-88.1)
RL.APL	21.4±4.4 (10.8-26.4)	18.9±4.3 (10.7-25.0)	21.2±4.3 (11.7-30.0)	25.2±5.5 (11.5-50.7)	21.3±11.2 (16.5-25.0)
RL.AMR	28.9±6.1 (16.0-30.0)	19.7±4.7 (10.7-26.6)	25.7±4.7 (12.7-36.6)	30.5±6.7 (12.4-47.3)	22.9±11.6 (15.0-28.3)
RL.PMR	40.2±12.5 (28.5-70.0)	30.1±9.9 (12.6-45.3)	38.1±9.9 (15.6-52.3)	47.9±11.6 (15.0-81.1)	31.8±8.2 (20.8-46.8)
AMR.PMR	140.3±20.2 (107.4-200.0)	148.8±20.6 (112.0-178.5)	151.8±20.6 (113.0-183.5)	150.1±20.3 (128.6-181.3)	141.8±19.6 (108.6-173.8)
DVL.APL	106.6±7.9 (97.0-125.0)	119.5±8.4 (107.8-131.2)	106.5±8.4 (100.8-119.2)	103.2±5.8 (90.0-122.1)	126.7*±9.6 (117.5-133.2)

RL.DVL, DVL.APL) differed significantly among the species (ANOVA with the post hoc Duncan test, $P < 0.05$). Characters AMR.DVL and AMR.APL significantly separated *A. dispar* from all the studied members of the IIRAS group including; *A. farsicus*, *A. isfahanensis*, *A. sophiae*, and *A. vladykovi*. Also, within the IIRAS group, RL.DVL and DVL.APL significantly separated *A. sophiae* and *A. vladykovi* from the other relatives, respectively (ANOVA with the post hoc Duncan test, $P < 0.05$) (Table 2). Canonical Discriminant Analysis (CDA) was based on all ten scale morphometric characters. The result indicated

separation between the brackish water species (*A. dispar*) and the IIRAS group; however, there was still some overlap between them (Fig. 6).

The average linkage dendrogram based on Euclidean distance was calculated for all ten scale characters. The resulting dendrogram categorized studied *Aphanius* species into two major clusters including "brackish water" and "IIRAS" (see the left side in Fig. 7). The brackish water cluster contains *A. dispar*, which was positioned as a basal cluster, and the IIRAS cluster contains the four other species.
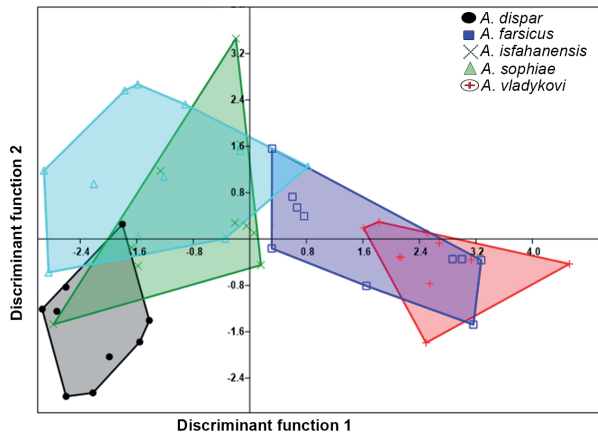
Figure 6. Canonical Discriminant Analysis (CDA) based on the ten morphometric characters of scales.
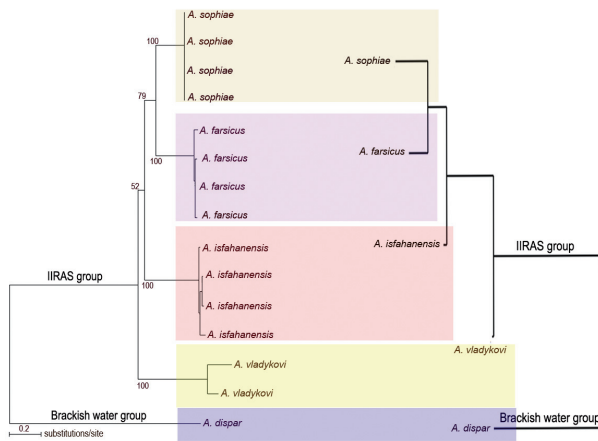

Figure 7. Maximum likelihood analysis based on the 15 cytochrome *b* sequences estimates phylogenetic relationships of the studied *Aphanius* species (left side), and the average linkage dendrogram based on the ten morphometric characters of scales (right side).

Geometric morphometric analyses

The mean scale shape among the four endemic Iranian inland and inland-related *Aphanius* species (IIRAS) and a native brackish water species, *A. dispar*, showed no overlap in CVA (P < 0.001). The four endemic *Aphanius* species could also be distinguished from each other with high reliability (P < 0.0001). The species were clearly separated with significantly different group means. Mahalanobis distance among the five species indicated high reliability based on the permutation test (P < 0.001). CVA

showed that the first two CVs captured 61.67, and 25.98% of the total shape variation, respectively. The general pattern of morphological differences described by these first two CVs was explored using transformation grids (Fig. 8a).

Shape changes associated with CV1 and CV2 were mainly due to the change in the left and right lateral tips of the anterior portion (LMs 1 and 3) and focus of the scale (LM5), while the shape changes associated with CV2 were mainly related to all of the landmarks with the exception of LMs 1 and 5.

In the UPGMA analyses, two major clusters were identified. The first cluster contained a single species of the brackish water group, *A. dispar*. The second cluster contained the four species of the IIRAS group. In the second cluster *A. sophiae* formed a sister group
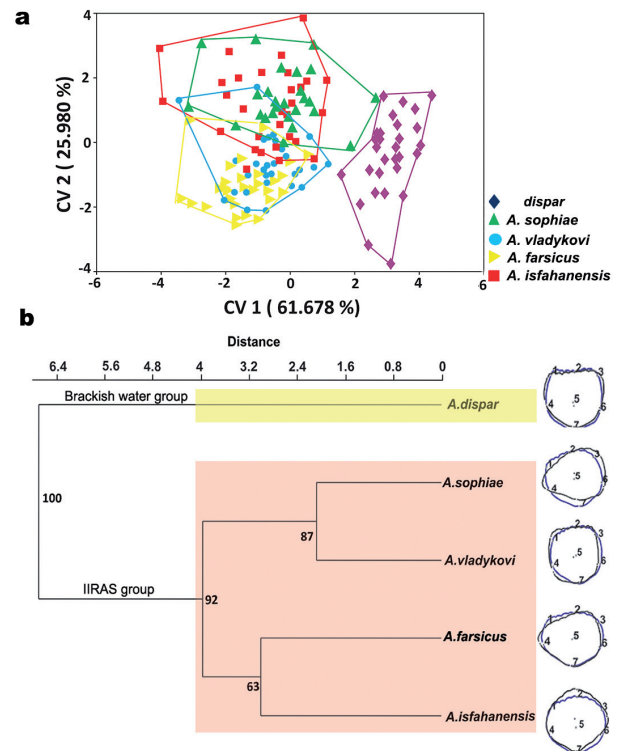

Figure 8. (a) Canonical Variate Analysis of the studied *Aphanius* species with landmark-based geometric morphometrics based on scale shape. General pattern of morphological differences described by the first two CVs was explored using transformation grids. The CV1 explained 61.67, and CV2 explained 25.98% of total shape variation respectively. (b) UPGMA cluster analysis performed on the matrix of shape distances (Euclidean Distances). Mean shape of scale based on the seven selected landmarks is shown for each species.

with *A. vladykovi,* and these together were a sister to another cluster containing *A. farsicus* plus *A. isfahanensis* (Fig. 8b).

Scanning electron microscopy (SEM)

The scanning electron microscopic investigation showed that there were tooth-like microscopic processes in the circuli of the rostral and lateral fields that can be seen only under high magnification and are known as "scalar denticles." These structures are usually distributed in minute or crowded spaces. The density of denticles was higher in the rostral field than the older circuli in the lateral field. The denticles also varied in shape. In the lateral field itself, the denticles were well developed in the anterior part, while they gradually reduced and disappeared in the most posterior parts. The comparative results on scalar denticles are as follows:

Aphanius farsicus – the scalar denticles were present in both the interradial space of the rostral and the anterior part of the lateral fields. The interradial denticles were spaced. Considering their morphology, the denticles were short and conical in both the rostral and lateral fields (Fig. 9a).

Aphanius isfahanensis – the scalar denticles were crowded, short, and rounded and tapered in the rostral field (Fig. 9b), while they were often short and rounded in the lateral fields (a few were long and conically pointed).

Aphanius sophiae – the scalar denticles were moderately spaced, short, and conical in the rostral filed. They were moderately spaced and conical in the lateral fields (Fig. 9c).

Aphanius vladykovi – the scalar denticles were moderately spaced and long and conically pointed in the rostral field. In the lateral fields, they often disappeared, and, if present, they were conical (Fig. 9d).

Aphanius dispar – the scalar denticles were moderately spaced, short, and roundly tapered in the rostral field (Fig. 9e). In the lateral fields, they often disappeared, and, if present, were conical (Fig. 9e).
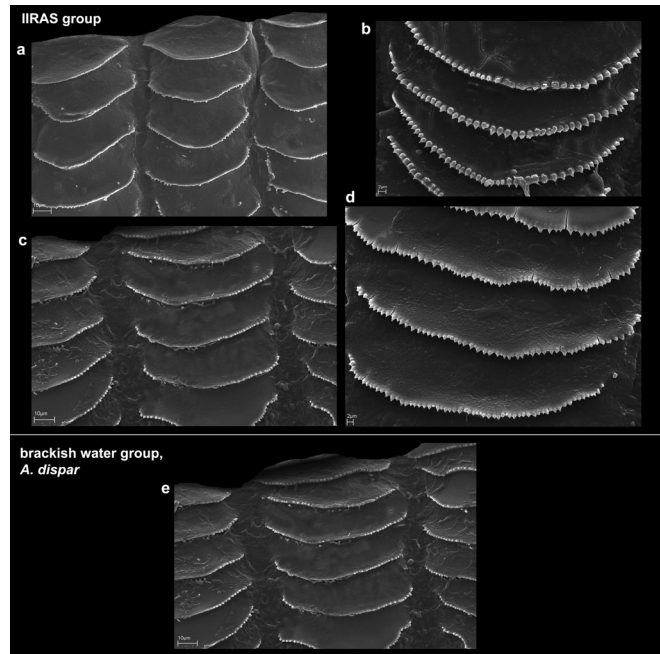

Figure 9. Patterns and shape of lepidonts present in anterior part of the lateral field of the body key scales shown for the IIRAS group (a-d) and brackish water group (e). They are spaced and short-conical in *A. farsicus* (a), crowded, often short and rounded, while few are long and pointed conical in *A. isfahanensis* (b), moderately spaced and conical in *A. sophiae* (c), often disappeared; if present conical *A. vladykovi* (d), crowded, short and rounded in *A. dispar* (e).

Phylogenetic relationships

Maximum-likelihood analysis indicated that the two groups (i.e., IIRAS and brackish water) of *Aphanius* species studied exhibited basal divergence, in which a brackish water species, *A. dispar*, formed a sister group with the IIRAS group. Among the IIRAS group, *A. vladykovi* showed the earlier divergence. *A. isfahanensis* was a sister to a clade containing *A. farsicus* from the Lake Maharlu Basin plus *A. sophiae* from the Kor River Basin (see the right side in Fig. 7).

Discussion

The objective of this study was to determine whether variation related to scale topology and scalar denticle morphology in the genus *Aphanius* was of taxonomic

and phylogenetic significance. The use and value of scale morphology in fish taxonomy was recognized more than one hundred years ago when Agassiz (1833-1843) classified fishes on the basis of four scale types (i.e., "Les Placoides" – spine-like denticles of enamel and dentine, "Les Ganoides" – thick plates of ganoine and bone, "Les Ctenoides" – thin plates with comb-like posterior borders, and "Les Cycloide" – thin plates with smooth borders). However, since then and especially after the introduction and development of scanning electron microscopy, scale morphology has became more important in fish systematics, and it has been used widely in the taxonomy and phylogeny of different marine and freshwater fish groups (e.g., Hughes 1981, Lippitsch 1990, 1992, Roberts 1993, Jawad 2005, Gholami et al. 2013, Teimori 2016).

Taxonomic values of scales in the genus *Aphanius* Nardo, 1827

The major difference among the scales of the studied *Aphanius* species was related to their topology, so they can be classified according to the pattern on the outer edge in the posterior part. The brackish water species *A. dispar* had spined scales with spinous-like structures on the outer edge of the posterior region, while inland and inland-related *Aphanius* species (IIRAS) had cycloid scales with smooth outer edges in the posterior region. By performing multivariate analysis (CDA) on the morphometric variables of scales and shape analysis (CVA) on the seven landmarks on the scales, we were able to separate the members of the IIRAS group from the brackish water group. However, the comparative results of the four data sets examined indicated similar patterns of among-species differentiation when we compared scale morphometry and the phylogenetic tree. Despite this, there were differences when we compared them with the geometric morphometric method. Further investigation also indicated differences regarding of the shape of the scalar denticles. Within the IIRAS group, two lepidont morphotypes were observed, i.e., pointed and conical. Conical is the most

common morphotype, which can be seen in the scales of *A. farsicus*, *A. sophiae*, and *A. vladykovi*. However, the most different types were observed in the scales of *A. isfahanensis* (pointed) from the Isfahan Basin, which was similar to the denticles observed in the scales of *A. dispar, a* brackish water species.

The taxonomic power of the scalar denticles, or lepidonts, has already been examined in fish taxonomy. Hollander (1986) documents the significance of characters related to the lepidonts in separating different poeciliid fishes. The taxonomic significance of lepidonts was also examined in cyprinodintid fishes and was found to be a useful tool for their taxonomy (Ferrito et al. 1998, 2001, 2003, Tigano et al. 2003). Ferrito et al. (2009) showed that the scale lepidonts of *A. vladykovi* (from the IIRAS group) and of *A. ginaonis* (from the brackish water group) were very similar. In spite of what Ferrito et al. (2009) reported, our results showed that lepidont morphology in the scales of *A. isfahanensis* is similar to that in *A. dispar*, and this results is supported by the recent study by Ba-Omar et al. (2013).

Based on the phylogenetic relationships among the studied *Aphanius* species, and also by taking into account the similar morphology seen in the scale lepidonts between *A. dispar* (a basal taxon in the phylogenetic tree) and *A. isfahanensis* (a member of the IIRAS group), it can be concluded that the presence of rounded lepidonts in members of the *Aphanius* species can be a primary state in their scales. Another possible explanation for the presence of rounded lepidonts in the *Aphanius* species is convergent evolution, which is the independent evolution of similar features (here rounded lepidonts) in species of different lineages. It should be noted that *A. dispar* and *A. isfahanensis* belong to two separate phylogenetic lineages (see the right side in Fig. 7).

Moreover, the general topology of the scale seems to vary among the studied *Aphanius* species. The brackish water *Aphanius* species in Iran (*A. dispar* and *A. ginaonis*) have spined scales (Esmaeili and Gholami 2007; this study), while species of the IIRAS group have cycloid scales (this study).

Additionally, the scales studied in *A. sophiae* and *A. isfahanensis* are polygonal and similar to those in *A. dispar*. *A. dispar* and *A. ginaonis* have a basal position in the phylogenetic trees of the genus *Aphanius* (Hrbek and Mayer 2003, Teimori et al. 2012). Therefore, it can be assumed that the polygonal state of the scales of *Aphanius* species is a primitive shared character state, which is probably inherited by the IIRAS group from the brackish water group.

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