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Osteology of Vertebral Column and Caudal Skeleton of Iranian Aphaniid Species (Teleostei: Cyprinodontiformes, Aphaniidae)

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ABSTRACT

In this study, a comparative analysis of the vertebral column and the caudal skeleton of 245 specimens of 10 *Aphanius* and three *Aphaniops* species was conducted based on X-ray imaging, and interspecific variation of these characters was examined. The vertebral bending index showed that straight and almost straight vertebral columns were more common in *Aphaniops* than *in the Aphanius* species. The numbers of abdominal and caudal vertebrae, principal caudal fin rays, and principal rays supported by the hypural plate were significantly lower in *Aphaniops* than in *Aphanius* species. Those species of *Aphanius* which were found in higher latitudes or altitudes had more vertebrae than the remaining *Aphanius* species and all members of *Aphaniops*, revealing the role of environmental factors. The number of preural (PU) vertebrae and the width of neural and haemal spines of preural vertebrae 2-4 were significantly higher in *Aphaniops* than in the *Aphanius* species. The conspicuous variations detected among localities of *A. arakensis* highlight the importance of more profound studies on the diversity of this species. In the genus *Aphaniops*, 17% of specimens showed a straight epural bone and 83% showed a sinusoidal bone. In comparison, *Aphanius* species showed that 91% and 7% of specimens displayed straight and sinuous shape epural bone, respectively. Such intra-species polymorphism of the epural character state was first reported for Aphaniid species. Hence, the previously proposed synapomorphy for the genus *Aphaniops* based on sinusoidal epural bones may need further investigation.

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Introduction

The tooth-carp fish family Aphaniidae Hoedeman 1949 is a large group of cyprinodontiform fish that are naturally distributed in the coastal and freshwater habitats along the Mediterranean Sea, Red Sea, Persian Gulf, Oman Sea, as well as in land-locked environments such as ponds and spring-stream systems in Turkey and Iran (Coad, 2000; Gholami *et al*., 2014; Reichenbacher *et al*., 2007; Teimori *et al*., 2018; Esmaeili *et al*., 2020). The species of the Aphaniidae family were classified into three distinct genera based on a recent molecular phylogeny, which could be divided into two groups (Esmaeili *et al*., 2020). The first group belongs to the genus *Aphanius* (the inland and inland-related species group) and the second group is the brackish water tooth-carps, consisting of two genera *Aphaniops* and *Paraphanius* (Esmaeili *et al*., 2020).

In previous studies, the taxonomy and phylogeny of Iranian Aphaniid species were studied using various methods such as multivariate analysis of morphometric and meristic traits, genetic analysis, coloration pattern, otolith morphology, and osteological study (Esmaeili *et al*., 2014; Teimori *et al*., 2017; Teimori *et al*., 2018; Charmpila *et al*., 2020; Esmaeili *et al*., 2020;

Teimori and Esmaeili, 2020). The vertebral column and caudal skeleton characters have been considered important sources of information for interpreting the systematic and phylogenetic relationships of actinopterygian fish (Arratia and Schultze, 1992). Characters of the caudal skeleton play a relevant role in studies on the systematics of cyprinodontiforms and other teleost fish and often provide useful phylogenetic information at different taxonomic levels (Costa, 1998, 2012; Parenti, 1981; Rosen, 1973, 1985). Costa (2012) described and compared the morphological traits of the caudal skeleton of the existing Cyprinodontiformes lineages and provided a series of synapomorphies for the further classification of killifish, including specimens of the genus *Aphanius* that were previously classified in Cyprinodontidae. Recently, Charmpila *et al*. (2020) reported differences in the skeleton and median fin osteology of four *Aphaniops* species and introduced some characteristics that can be useful for species identification. In their comprehensive study, Teimori and Esmaili (2020), investigated and described differences between the axial and caudal skeleton characters of some species of the Aphaniidae family (including six *Aphanius* and two *Aphaniops* species from the Iranian Basin that include these species as well). They supported the assumption about introducing sinuous-shaped epural bone in all species of the genus *Aphaniops* as the first detected morphological synapomorphy for the "inner" clade of *Aphaniops* (Charmpila *et al*., 2020).

In this study, the interspecific differences in some important characters of the vertebral column and caudal skeleton of 10 Iranian *Aphanius* and 3 *Aphaniops* species from the Aphaniidae family are investigated based on more specimens to probe if these traits are useful for further taxonomic or phylogenetic analysis.

Materials and Methods

Studied specimens

In this study, a total of 245 specimens from 10 species of the genus *Aphanius* and 3 species of the genus *Aphaniops* were studied (Table 1). Osteological, meristic, and morphometric characters were studied based on digital X-ray images that were taken with a Faxitron Bioptics (LLC-Vision NDT ver. 2.2.5, 45k.v. and 30 s), housed in the SNSB-Zoological State Collection, Munich, for all specimens and served as the basis for the osteological study.

Osteology

Figure 1 shows an overview of an X-ray image of *Aphanius sophiae* to illustrate the osteological structure of the vertebral column and caudal skeleton. The terms used in this study follow Schultze and Arratia (2013). The curve length of the vertebral column (D) and linear distance between the first vertebrae and terminal centrum (L) were measured to quantify the shape of the vertebral column (Figure 2). Since fish with curved vertebral columns typically exhibit longer curve lengths than the linear length of the vertebral column, the Vertebrae Bending Index (VBI) was measured (Shao *et al*., 2018). This method is more precise and reliable than the visual inspection used in earlier studies for defining variations in the shape of the vertebral column. The VBI was calculated as the vertebral curve length (D) divided by vertebral linear length (L). All measurements were done using ImageJ, version 1.53.

Vertebrae counts comprise abdominal and caudal vertebrae; the latter includes the terminal centrum. Following Schultze and Arratia (2013), caudal ray counts include principal rays of the caudal fin that are all the segmented and branched rays plus normally one unbranched but segmented ray located at the leading margin in each lobe of the fin; they are associated with endoskeletal elements (*e.g.*, hypurals, haemal spines of preural centrum 2). In addition, the segmented and branched principal rays that are only supported by the hypural plate, are separately counted (Figure 1b) (Schultze and Arratia, 2013). According to Costa (2012), in Cyprinodontiforms, a single and elongated epural bone has two distinct shapes: straight and sinuous shape. The straight epural is a blade-like bone with a flat core abruptly narrowing ventrally and a thin flap on the anteroventral portion. In the sinuous shape, the short and narrow core part of the epural is restricted to its dorsal portion, and the whole bone shows a slightly sinuous shape.

Table 1. Studied specimens.

Genus and Species	Region/Basis	N (♂/♀)	Standard Lengths	
			Males	**Females**
Genus: *Aphanius*				
A. arakensis	Arak, Namak Basin	6/14	22.35-30.40 (25.96±3.4)	22.69-35.35 (28.61±3.65)
	Karaj, Namak Basin	9/4	19.18-36.79 (25.83±5.1)	22.05-44.21 (29.94±9.9)
	Varamin, Namak Basin	4/3	25.89-35.10 (29.84±4.42)	33.62-39.07 (35.84±2.85)
A. darabensis	Darab, Hormuzgan Basin	0/5	-	21.59-25.23 (23.16±1.41)
A. farsicus	Babunak spring, Maharlu Lake System	8/19	15.03-25.28 18.11±3.36	14.46-34.25 19.30±5.32
A. isfahanensis	Varzaneh, Esfahan Basin	3/3	19.58-20.31 (19.9±0.37)	21.18-26.92 (23.42±3.07)
	Zayandehroud, Esfahan Basin	7/4	27.72-31.5 (29.11±1.35)	30.51-36.94 (34.22±2.860
	Izeh, Esfahan Basin	3/3	24.35-28 (26.18±1.82)	20.68-26.48 (23.95±2.96)
	Gavkhuni, Esfahan Basin	1/1	35.74	42.48
	Dastjerd, Esfahan Basin	0/5	-	25.18-29.18 (26.22±1.68)
A. kavirensis	Damghan, Kavir Basin	0/3	-	28.08-36.61 (31.49±4.5)
A. mesopotamicus	Shadegan, Tigris Basin	4/4	19.87-24.48 (22.10±2.2)	20.48-25.23 (22.56±1.83)
A. pluristriatus	Khonj, Mond River Basin	6/4	21.23-30.67 (23.76±3.46)	17.67-21.42 (19.4±1.61)
A. shirini	Khosroshirin, Kor River Basin	6/3	20.32-27.05 (23.94±3.01)	23.32-34.55 (27.18±6.38)
A. sophiae	Ghadamgah, Kor River Subsystem	13/16	17.9-30.15 (22.86±3.33)	20.86-39.40 (27.02±4.6)
A. vladykovi	Chaghakhor, Tigris Basin	5/7	18.61-24.59 (22.25±2.5)	19.71-27.7 (23.73±2.66)
	Ghandoman, Tigris Basin	3/0	20.48-21.67 (21.11±0.59)	-
Genus: *Aphaniops*				
A. ginaonis	Genow, Hormuzgan Basin	8/5	20.40-26.46 (23.03±2.53)	16.28-20.63 18.94±1.66
A. stoliczkanus	5km Mirahmad, Helle Basin	1/3	23.65	29.39-29.78 (29.58±0.27)
	Ilam-Sartang, Tigris Basin	6/0	33.76-38.64 (36.24±1.85)	-
A. hormuzensis	Mehran, Hormuzgan Basin	1/5	25.76	20.18-26.31 (22.47±2.30)
	Minab road, Hormuzgan Basin	0/7	-	24.58-30.45 (26.33±2.01)
	Mehran-Rudan, Hormuzgan Basin	5/3	20.27-33.43 (25.59±5.09)	27.30-27.65 (27.51±0.18)
	Khurgu, Hormuzgan Basin	3/3	27.47-28.99 (28.35±0.79)	25.27-33.42 (29.63±4.1)
	Mehran-Kukherd, Hormuzgan Basin	6/6	25.28-37.34 (27.94±4.65)	27.51-31.65 (29.24±1.51)
	Kahurestan, Hormuzgan Basin	4/0	20.49-29.68 24.74±4.63	-
	Faryab, Hormuzgan Basin	1/2	27.02	20.78-23.14 (21.96±1.66)

N = Number of specimens, standard lengths are given as ranges and means ± standard deviation.

In this study, we use the term 'Modified Caudal' (MC) vertebra (Charmpila *et al.*, 2020). This term is used for each vertebra that has a visible modification of its neural and/or haemal spine in comparison to a 'usual' caudal vertebra, like a preural vertebra. However, it differs from a PU vertebra, as it is not involved in caudal ray support (Figure 1b) (Charmpila *et al*., 2020).

As the width of the spines of the preural vertebrae PU2-PU4 is considered an important characteristic in phylogenetic analysis within Cyprinodontiformes, widths of spines attached to PU2, PU3, and PU4 were measured based on the width of the most distal part of the respective neural or haemal spine (Figure 1b). In the case that the distal tip of a spine was obscured by caudal fin rays, its width was measured just before these rays (Altner and Reichenbacher, 2015).

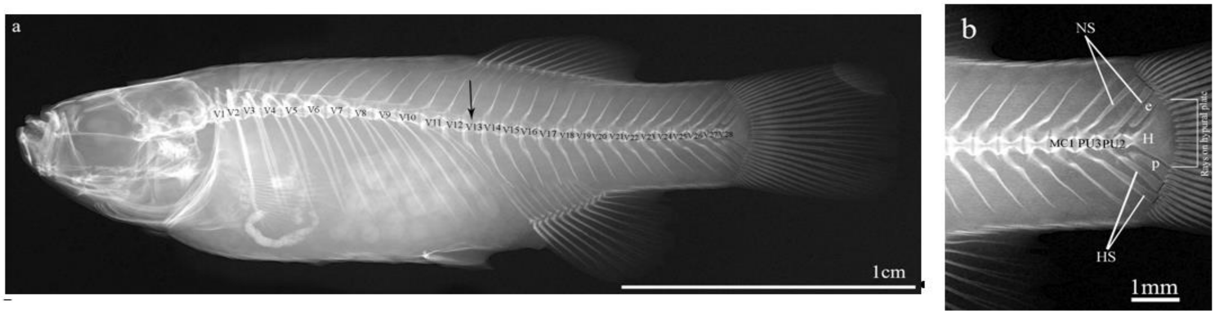

Fig. 1. X-ray image of *Aphanios sophiae* shows a whole specimen(a) and caudal (b). e, epural; H, hypural; HS, Haemal Spines; NS, Neural Spines; p, parhypural; PU2-3, PU centra; MC, Modified Caudal vertebra. Black lines show the measurements of neural and haemal spines of PU vertebrae. The arrow shows the first caudal vertebrae.

Statistical analyses of the morphometric and meristic data were carried out by SPSS, version 26.00. Meristic count data between the two groups were compared by Mann–Whitney non-parametrical U-tests ($p < 0.05$). Kruskal–Wallis test ($p < 0 .05$) was performed for multiple group comparisons.

The relationship between the widths of the spines of the PU2–PU4 and fish total length was checked using Pearson's correlation coefficient (r) to find a significant correlation between these variables ($p < 0.05$), except for the widths of the neural spines of the PU3. To eliminate this effect, the standard residuals from a linear regression of each measurement on total length were calculated for the whole data (Reist, 1985). The size-corrected measurements were screened for normality and homogeneity of variance using Kolmogorov-Smirnov normality tests and Levene's tests, respectively. The measurements were normally distributed ($p > 0.05$) and they showed homogeneity of variance ($p > 0.05$). As a result, it is possible to use parametric tests (T-test, $p < 0.05$, ANOVA, < 0.05) for comparing the groups.

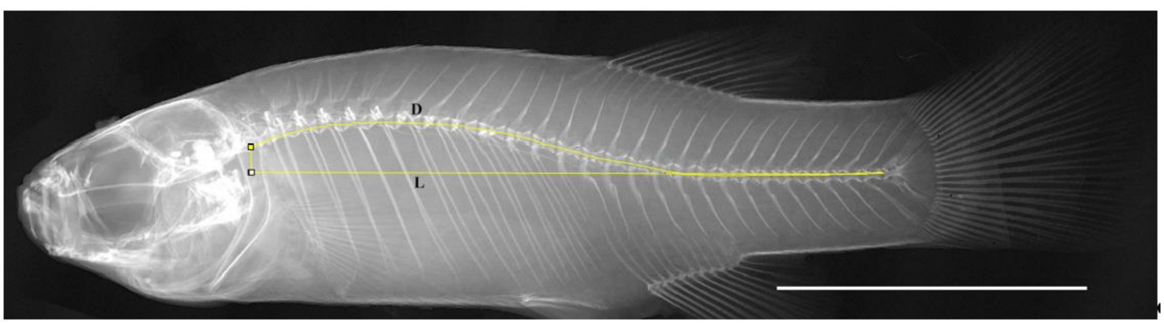

Fig. 2. Vertebrae bending index measurement. The Vertebrae Bending Index (VBI) = curve length of the vertebral column (D) / linear distance between the first vertebrae and terminal centrum (L). Scale bar= 1cm.

Results

No significant differences were observed between the sexes in any species (Man Whitney; P> 0.05); therefore, samples were pooled from both sexes.

Vertebral column

The X-ray images clearly showed variability in the shape of the vertebral column in the studied species (Figure 3). Three forms are excised; curved, straight, and almost straight vertebral columns. This variability was quantified by calculating the VBI. For comparison, we determined VBI = 1 as "straight", VBI ≥1.01 as "curved", and 1< VBI < 1.01 as "almost straight". In 72% of *Aphanius* specimens, the vertebral columns were curved, while in 18% and 10% of specimens, the vertebral columns were straight and almost straight, respectively. In the genus *Aphaniops*, 50% of specimens showed curved vertebral columns and in 26% and 24% of the specimens, the vertebral columns were straight and almost straight, respectively (Table 2).

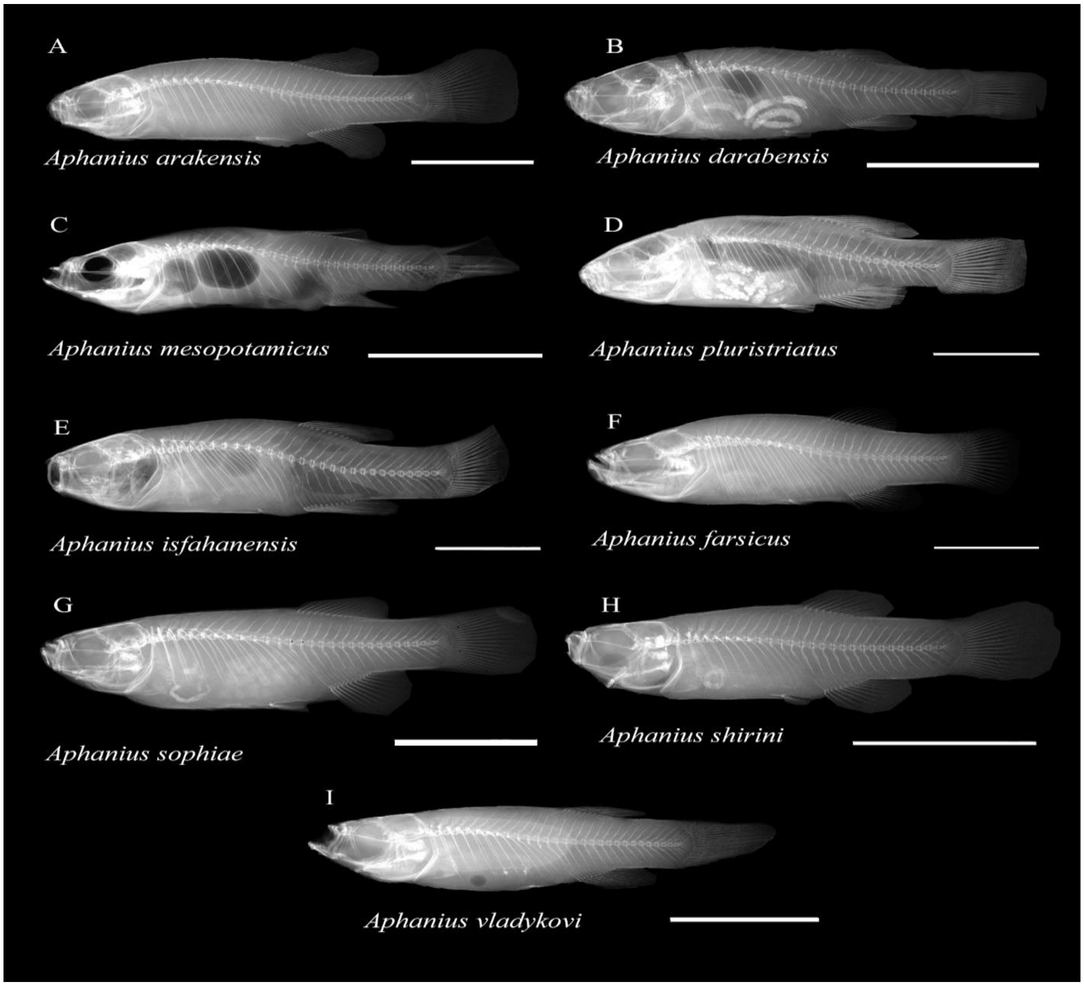

Fig. 3. X-ray image of *Aphanius* species showing the varying extent of curvature of the vertebral column: A, B, C, D, E, F, curved; G, almost straight; H, I, straight. Scale bars 1 cm.

The VBI was significantly lower in *A. arakensis* from Varamin and *A. ginaonis* concerning other species (Kruskal-Wallis, p<0.05). In all species of the genus *Aphanius,* the curved vertebral columns are more common than straight and almost straight shapes, except for the specimens of *A. arakensis* from Varamin and *A. kavirensis* (Table 2). Regarding the genus *Aphaniops,* while the straight and almost straight shape were common in *A. ginaonis,* the curved vertebral columns were more common among the specimens of *A. stoliczkanus* except for the specimens from Ilam-Sartang and *A. hormuzensis* except for specimens from Faryab (Figure 4).

The total number of vertebrae in the studied species of the genus *Aphanius* varied from 26 to 30, among which 11-13 were abdominal vertebrae and 14-18 were caudal vertebrae (Table 3). In the genus *Aphaniops*, the total number of vertebrae varied from 26 to 29, among which 10-13 were abdominal vertebrae and 14-17 were caudal vertebrae. The mean values of abdominal (Mann–Whitney, p < 0.05) and caudal vertebrae (Mann–Whitney, p < 0.05), were significantly lower in *Aphaniops* than those of *Aphanius* species. We did not find any significant differences between the localities in *A. arakensis* (Kruskal-Wallis, p>0.05) in the number of abdominal and caudal vertebrae. The mean values of the abdominal vertebrae in the specimens of *A. arakensis* from Arak were significantly higher than those of *A. darabensis, A. farsicus, A. isfahanensis* (except Dastjerd), *A. kavirensis*, *A. mesopotamicus, A. pluristriatus* and *A. shirini* (Mann–Whitney, p <0.05 for all pairwise comparison).

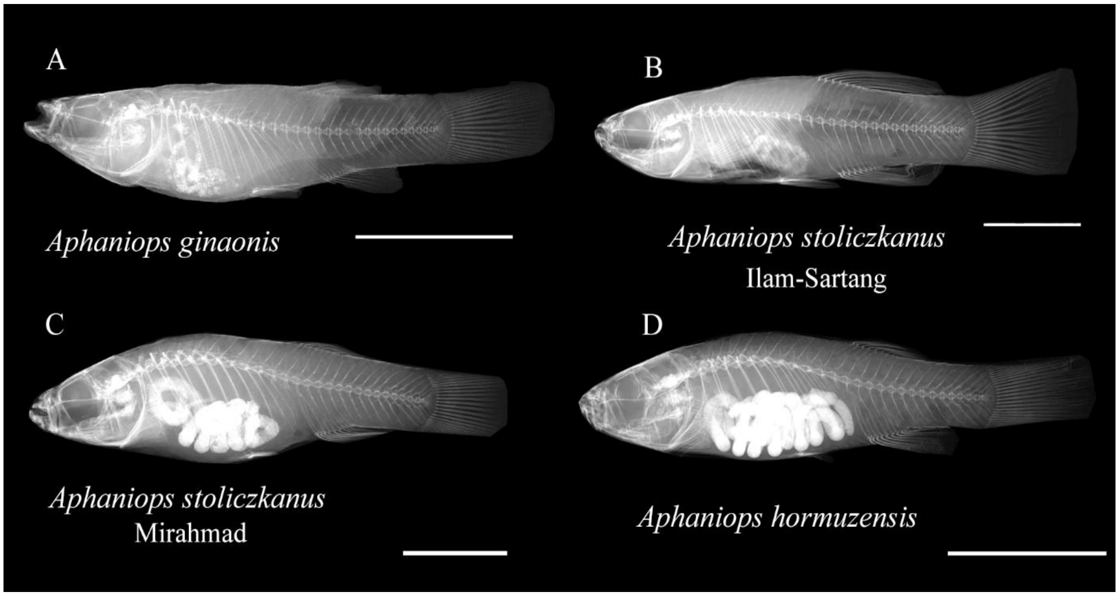

Fig. 4. X-ray image of *Aphaniops* species showing the varying extent of curvature of the vertebral column: A, B, straight; C, D, curved, Scale bars= 1 cm.

Table 2. Percentage of different shapes of vertebrae column based on the VBI among the studied Aphaniid species.

Genus and Species	Region/Basis	VBI≥ 1.01 Curved	1<VBI<1.01 Almost Straight	VBI= 1.0 Straight
Genus: *Aphanius*				
A. arakensis	Arak, Namak Basin	100	-	
	Karaj, Namak Basin	75	-	25
	Varamin, Namak Basin	17	50	33
A. darabensis	Darab, Hormuzgan Basin	80	-	20
A. farsicus	Babunak spring, Maharlu Lake System	70		30
A. isfahanensis	Varzaneh, Esfahan Basin	100	-	
	Zayandehroud, Esfahan Basin	50	30	20
	Izeh, Esfahan Basin	100	-	
	Gavkhuni, Esfahan Basin	50	50	
A. kavirensis	Damghan, Kavir Basin	30	-	70
A. mesopotamicus	Shadegan, Tigris Basin	86		14
A. pluristriatus	Khonj, Mond River Basin	75	-	25
A. shirini	Khosroshirin, Kor River Basin	67	16	17
A. sophiae	Ghadamgah, Kor River Subsystem	67	11	22
A. vladykovi	Chaghakhor, Tigris Basin	67	16	17
Genus: *Aphaniops*				
A. ginaonis	Genow, Hormuzgan Basin	11	22	66
A. stoliczkanus	5km Mirahmad, Helle Basin	100		
	Ilam-Sartang, Tigris Basin	-	50	50
A. hormuzensis	Mehran, Hormuzgan Basin	60		40
	Minab road, Hormuzgan Basin	71	29	
	Mehran-Rudan, Hormuzgan Basin	50	10	40
	Khurgu, Hormuzgan Basin	80		20
	Mehran-Kukherd, Hormuzgan Basin	80	20	
	Faryab, Hormuzgan Basin	33	66	

Table 3. Frequencies of counts of meristic characters among the studied Aphaniid species.

Genus and Species	Region/Basis	Abdominal Vertebrae					Caudal Vertebra						Total Vertebra					
		10	**11**	**12**	**13**	**Mean ± SD**	**14**	**15**	**16**	**17**	**18**	**Mean ± SD**	**26**	**27**	**28**	**29**	**30**	**Mean ± SD**
Genus: *Aphanius*																		
A. arakensis	Arak, Namak Basin	-	-	7	13	12.65± 0.4	-	1	12	7	-	16.30± 0.5	-	-	3	16	1	28.90± 0.4
	Karaj, Namak Basin	-	-	8	5	12.38± 0.5	-		8	4	1	16.46± 0.6	-	-	3	9	1	28.85± 0.5
	Varamin, Namak Basin	-	1	4	2	12.14± 0.7	-	1	3	3	-	16.29± 0.7	-	-	4	3	-	28.43±0.5
A. darabensis	Darab, Hormuzgan Basin	-	1	4		11.80± 0.45	2	3		-	-	14.60± 0.5	3	2	-	-	-	26.40± 0.5
A. farsicus	Babunak spring, Maharlu Lake System	-	6	22	-	11.79± 0.41	-	14	15	-	-	15.52± 0.5	4	12	12	-	-	27.29± 0.7
A. isfahanensis	Varzaneh, Esfahan Basin	-	3	3	-	11.5± 0.5	-		4	2	-	16.33± 0.5	-	2	3	1	-	27.82± 0.7
	Zayandehroud, Esfahan Basin	-	2	8	1	11.91± 0.5	-		8	3	-	16.27± 0.4	-	1	7	3	-	28.18± 0.6
	Izeh, Esfahan Basin	-	2	3	1	11.8± 0.7	-	2	2	2	-	16± 0.9	-	1	5	-	-	27.83± 0.4
	Gavkhuni, Esfahan Basin	-	-	1	1	11.5± 0.7	-		2	-	-	16	-	-	2	-	-	28
	Dastjerd, Esfahan Basin	-	-	4	1	12.2± 0.4	-	2	3	-	-	15.60± 0.5	-	1	4	-	-	27.80± 0.4
A. kavirensis	Damghan, Kavir Basin	-	-	3	-	12	-	1	2	-	-	15.67± 0.5	-	1	2	-	-	27.61± 0.5
A. mesopotamicus	Shadegan, Tigris Basin	-	1	7	-	11.88± 0.3	-	5	2	1	-	15.50± 0.7	1	4	2	1	-	27.83± 0.9
A. pluristriatus	Khonj, Mond River Basin	-	-	10	-	12	-	8	2	-	-	15.20± 0.4	-	8	2	-	-	27.20± 0.4
A. shirini	Khosroshirin, Kor River Basin	-	1	8	-	11.89± 0.3	-		6	3	-	16.33± 0.5	-	-	7	2	-	28.22± 0.4
A. sophiae	Ghadamgah, Kor River Subsystem	-	-	21	10	12.31± 0.4	-	3	17	11	-	16.26± 0.6	-	2	9	20	-	28.58± 0.6
A. vladykovi	Chaghakhor, Tigris Basin	-	-	11	1	12.08± 0.2	-	1	8	3	-	16.17± 0.5	-	1	7	4	-	28.25± 0.6
	Ghandoman, Tigris Basin	-	-	2	1	12	-		2	1	-	16.33± 0.5	-	-	1	2	-	28.67± 0.5
Genus: *Aphaniops*																		
A. ginaonis	Genow, Hormuzgan Basin	1	9	2	-	11.08± 0.5	-		9	2	-	16.18± 0.4	-	1	6	4	-	27.27± 0.6
A. stoliczkanus	5km Mirahmad, Helle Basin	-	-	4	-	12	1	2	-	-	-	14.67± 0.5	1	2	-	-	-	26.67± 0.5
	Ilam-Sartang, Tigris Basin	-	1	5	-	11.83± 0.4	-	3	3	-	-	15.5± 0.5	-	4	2	-	-	27.33± 0.5
A. hormuzensis	Mehran, Hormuzgan Basin	-	2	3	-	11.6± 0.5	-	5	-	-	-	15	2	3	-	-	-	26.60± 0.5
	Minab road, Hormuzgan Basin	-	-	3	4	12.57± 0.5	1	5	1	-	-	15± 0.5	-	3	4	-	-	27.57± 0.5
	Mehran-Rudan, Hormuzgan Basin	-	-	7	1	12.13± 0.3	-		8	-	-	16	-	7	1	-	-	28.13± 0.3
	Khurgu, Hormuzgan Basin	-	1	5	-	11.8± 0.4	-	4	1	-	-	15.2	-	4	1	-	-	27.20± 0.4
	Mehran-Kukherd, Hormuzgan Basin	-	-	10	2	12.17± 0.3	4	8	-	-	-	14.67± 0.5	3	8	1	-	-	26.83± 0.5
	Kahurestan, Hormuzgan Basin	-	1	1	-	11.5± 0.7	-	2	-	-	-	15	1	1	-	-	-	26.50± 0.7
	Faryab, Hormuzgan Basin	-	1	1	1	12	1	2	-	-	-	14.67± 0.5	1	2	-	-	-	26.67± 0.5

Table 4. Percentage of a different number of principal caudal fin rays and segmented and branched fin rays on the hypural plate in the studied Aphaniid species.

Genus and Species	**Region/Sasis**	**Number of Principal Caudal Fin Rays**							**Number of Rays on Hypural Plate**					
Genus: *Aphanius*		16	17	18	19	20	21	22	8	9	10	11	12	13
A. arakensis	Arak, Namak Basin	-	14	-	43	-	29	14	-	-	70	30	-	-
	Karaj, Namak Basin	11	45	33	11	-	-	-	-	-	92	7	-	-
	Varamin, Namak Basin	-	-	20	60	-	20	-	-	-	-	-	71	29
A. darabensis	Darab, Hormuzgan Basin	-	-	50	50	-	-	-	-	-	60	20	20	-
A. farsicus	Babunak spring, Maharlu Lake System	-	-	-	-	-	-	-	-	4	32	57	7	-
A. isfahanensis	Varzaneh, Esfahan Basin	-	-	-	-	-	-	-	-	-	17	83	-	-
	Zayandehroud, Esfahan Basin	-	40	20	40	-	-	-	-	-	9	67	18	9
	Izeh, Esfahan Basin	-	50	25	25	-	-	-	-	-	-	50	50	-
	Gavkhuni, Esfahan Basin	-	-	50	50	-	-	-	-	-	-	50	50	-
	Dastjerd, Esfahan Basin	-	-	100	-	-	-	-	-	-	25	50	25	-
A. kavirensis	Damghan, Kavir Basin	-	-	-	33	34	33	-	-	-	-	33	67	-
A. mesopotamicus	Shadegan, Tigris Basin	-	-	-	50	50	-	-	-	-	37	51	12	-
A. pluristriatus	Khonj, Mond River Basin	16	68	-	16	-	-	-	-	-	60	40	-	-
A. shirini	Khosroshirin, Kor River Basin	-	-	50	50	-	-	-	-	-	-	45	22	33
A. sophiae	Ghadamgah, Kor River Subsystem	-	11	11	34	22	-	22	-	-	19	56	19	6
A. vladykovi	Chaghakhor, Tigris Basin	-	-	-	38	50	12	-	-	-	33	58	-	9
	Ghandoman, Tigris Basin								-	-	-	33	67	-
Genus: *Aphaniops*														
A. ginaonis	Genow, Hormuzgan Basin	-	25	25	50	-	-	-	27	73	-	-	-	-
A. stoliczkanus	5km Mirahmad, Helle Basin	-	100	-	-	-	-	-	-	67	33	-	-	-
	Ilam-Sartang, Tigris Basin	20	40	40	-	-	-	-	-	16	83	-	-	-
A. hormuzensis	Mehran-Rudan, Hormuzgan Basin	-	-	100	-	-	-	-	-	50	50	-	-	-
	Kahurestan, Hormuzgan Basin	-	-	-	-	-	-	-	-	100	-	-	-	-
	Mehran, Hormuzgan Basin	20	80	-	-	-	-	-	-	60	40	-	-	-
	Minab road, Hormuzgan Basin	50	50	-	-	-	-	-	17	83	-	-	-	-
	Khurgu, Hormuzgan Basin	-	50	-	-	50	-	-	-	60	40	-	-	-
	Mehran-Kukherd, Hormuzgan Basin	9	27	46	18	-	-	-	-	67	33	-	-	-
	Faryab, Hormuzgan Basin	-	-	-	-	-	-	-	-	100	-	-	-	-

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The comparison of the mean values of the caudal vertebrae showed that the specimens of *A. arakensis*, *A. isfahanensis* (except Dastjerd), *A. shirini*, *A. sophiae*, and *A. vladykovi* had significantly higher vertebrae than *A. darabensis*, *A. farsicus*, *A. mesopotamicus* and *A. pluristriatus* (Mann-Whitney, p <0.05 for all pairwise comparison). In the genus *Aphaniops*, no significant differences were observed in the mean values of the vertebrae among the species (Kruskal-Wallis, p>0.05). The mean values of the abdominal vertebrae in *A. ginaonis* were significantly lower than those of *A. stoliczkanus* and *A. hormuzensis* from Mehran-Rudan, Minab road and Mehran-Kukherd (Mann–Whitney, p < 0.05 for all pairwise comparison). The mean values of caudal vertebrae in *A. ginaonis* were significantly higher than those of *A. stoliczkanus* (Mann-Whitney, p < 0.05) and *A. hormuzensis* from all localities except Mehran-Rudan (Mann-Whitney, p < 0.05).

Caudal skeleton

Caudal fin

The number of the principal caudal fin rays was significantly higher in the genus *Aphanius* (16 - 22; 18.72± 1.47) than in *Aphaniops* (16-20; 17.53± 1.01) (Mann–Whitney, p < 0.05) (Table 4). In addition, the number of the segmented and branched principal rays supported only by the hypural plate was significantly higher in the genus *Aphanius* (9-13; 10.91±0.84) than in *Aphaniops* (8-10; 9.25± 0.59) (Mann–Whitney, p< 0.05) (Table 4). The number of the principal caudal fin rays in the specimens of *A. arakensis* from Karaj was significantly lower than those of Arak (Mann-–Whitney, p< 0.05) and Varamin (Mann–Whitney, p< 0.05). The number of principal caudal fin rays in the specimens of *A. arakensis* from Karaj and *A. pluristriatus* were significantly lower than the rest species of the genus *Aphanius* (Kruskal-Wallis, p< 0.05). In the genus *Aphaniops*, no significant differences were observed in the number of the principal caudal fin rays among the species (Kruskal-Wallis, p> 0.05).

In the genus *Aphanius*, the specimens of *A. arakensis* from Varamin and Karaj had higher and lower principal fin rays on the hypural plate, respectively. The specimens of *A. arakensis* from Varamin, *A. shirini*, and *A. kavirensis* had significantly higher principal fin rays on the hypural plate than those of *A. darabensis*, *A. farsicus*, *A. mesopotamicus*, and *A. pluristriatus* (Mann–Whitney, p<0.05 for all pairwise comparison).

In the genus *Aphaniops*, the specimens of *A. ginaonis* and *A. stoliczkanus* had lower and higher principal fin rays on the hypural plate among *Aphaniops* species, respectively. The number of the principal fin rays on the hypural plate in *A. ginaonis* was significantly lower than in *A. hormuzensis* (Mann–Whitney, p < 0.05).

Epural

In the studied species, the straight shape of the epural was seen in two forms. In one type, the distal region was wide and truncated in shape and the proximal part was relatively wide as well (Figure 5A). In the other type, the distal end was wide, however, the proximal part was distinctively narrow and ridge-like (Figure 5B). While in the *Aphanius* species, the epural with a relatively wide proximal part had a higher frequency than the other two epural shapes, in *Aphaniops*, the epural with sinuous shape had a higher frequency than the other two epural shapes (Figure 5C).

In both genera *Aphanius* and *Aphaniops*, the epural was polymorphic (Table 5). In the genus *Aphanius*, 71% of the specimens showed an epural with a relatively wide proximal part (Figs. 6A, C, G, I) and 20% of the specimens showed an epural with a narrow proximal part (Figures 6H, J). The remaining 7% of specimens showed a sinusoidal shaped epural (Figures 6B, D, F).

In the genus *Aphaniops*, 14% of the specimens showed an epural with a relatively, wide proximal portion (Figures 7A, C, D, E, H) and 3% of specimens showed an epural with a narrow proximal part. The remaining 83% of specimens displayed an epural with a sinuous shape (Figures 7 B, F, G, I). In specimens of *A. stoliczkanus* selected from Sartang, there were pointed projections in the middle of the epural bone in five of the six specimens (Figure 7G).

PU vertebrae 2-4 and modified caudal vertebrae

In the studied Aphaniid species, there were three, two, or less frequently, four PU vertebrae in the caudal skeleton (Figures 6, 7). These vertebrae are distinct from the remaining vertebrae since the PU vertebrae have longer neural and haemal spines that support the caudal fin rays. The number of PU vertebrae in *Aphaniops* (2-4, 3± 0.25) was significantly higher than those of *Aphanius* (2-3, 2.35± 0.48) (Mann-Whitney, $p< 0.05$) (Table 6). The number of PU vertebrae in *A. arakensis* from Varamin was significantly higher than those of *A. darabensis* (Mann-Whitney, $p < 0.05$) and *A. farsicus* (Mann-Whitney, $p < 0.05$). Among *Aphaniops* species, the number of PU vertebrae was significantly higher in *A. ginaonis* (Kruskal-Wallis, $p<0.05$).

Anterior to the PU vertebrae, one or two modified caudal vertebrae may be presented in both genera. A comparison of the species showed that only the specimens of *A. shirini* had significantly lower modified caudal vertebrae than the other *Aphanius* and *Aphaniops* species (Mann–Whitney, $p <0.05$ for all pairwise comparisons) (Table 6).

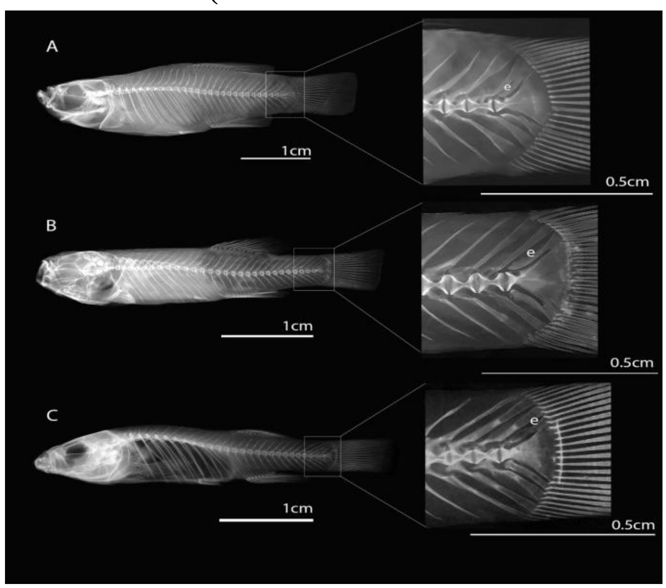

Fig. 5. X-ray image of *Aphanius* species showing the different epural shapes. A) epural with the wide distal region and relatively wide proximal part in *A. arakensis*; B, epural with the wide distal region and narrow and ridge-like proximal part in *A. isfahanensis*; C, epural with sinuous shape in *A. pluristriatus*.

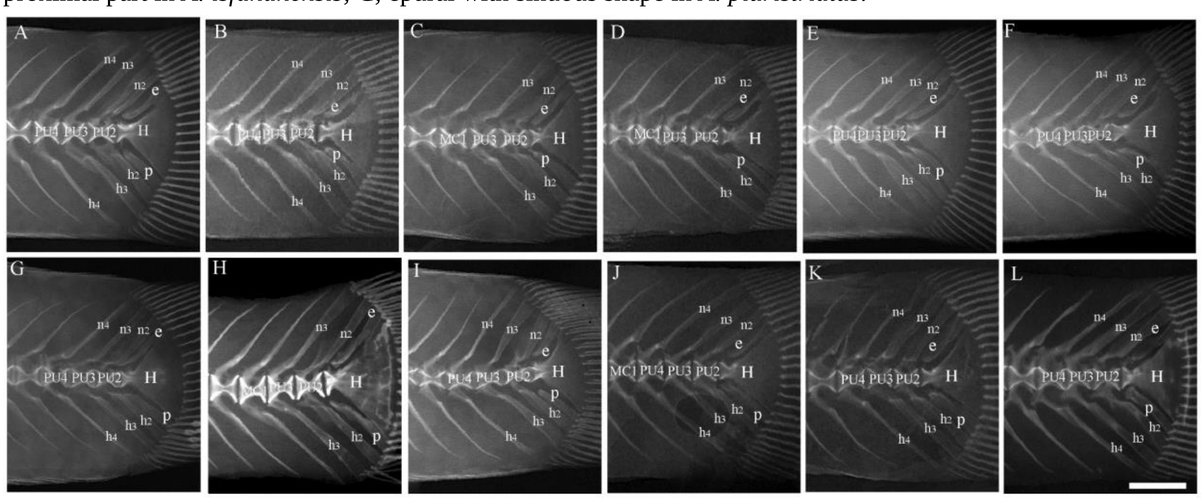

Fig. 6. Caudal skeleton of *Aphanius* species. A-B: *A.arakensis*; C-D: *A. darabensis*; E-F: *A. farsicus*; G-H: *A. isfahanensis*; I: *A. kavirensis*; J: *A. mesopotamicus*; K-L: *A. pluristriatus.* Scale bar 1 mm.

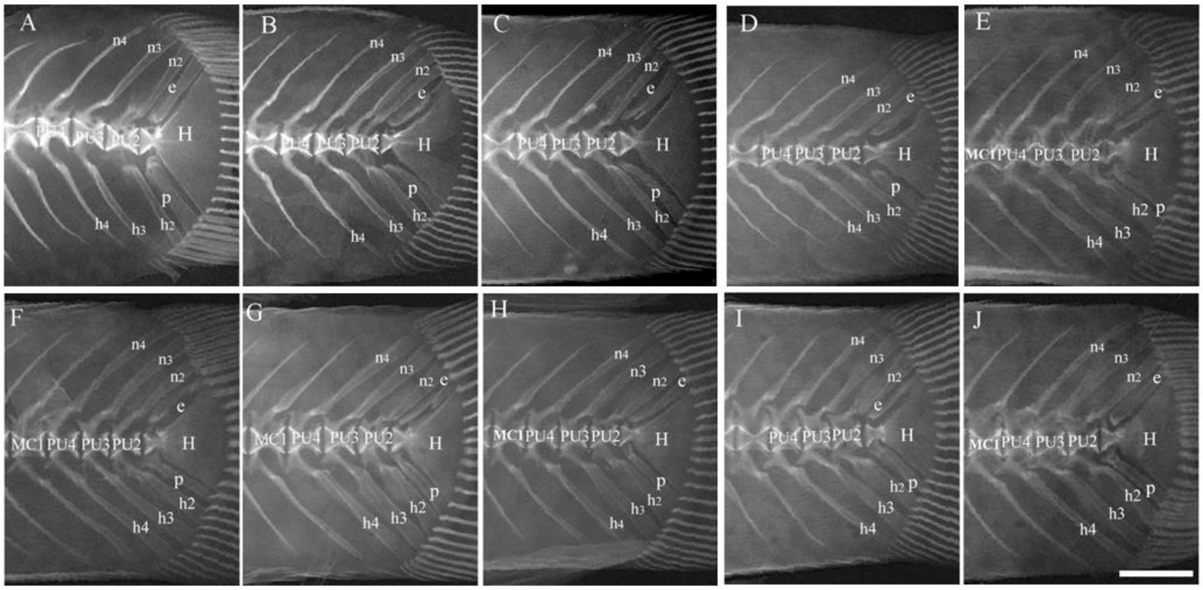

Fig. 7. Caudal skeleton of *Aphanius* and *Aphaniops* species: A-B) *A. shirini*; C) *A. sophiae*; D) *A. vladykovi*; E-F) *A. ginaonis*; G-H) *A. stoliczkanus* from Sartang; I-J) *A. hormuzensis*. Scale bar 1 mm.

Table 5. Percentage of different shapes of epural bone among the studied Aphaniid species.

Genus and Species	Region/Basis	Shape of Epural		
		Straight, Proximal Part Wide	**Straight, Proximal Part Narrow**	**Sinuous**
Genus: *Aphanius*				
A. arakensis	Arak, Namak Basin	70	20	5
	Karaj, Namak Basin	61	15	24
	Varamin, Namak Basin	57	43	-
A. darabensis	Darab, Hormuzgan Basin	40	40	20
A. farsicus	Babunak spring, Maharlu Lake System	79	21	-
A. isfahanensis	Varzaneh, Esfahan Basin	50	50	-
	Zayandehroud, Esfahan Basin	36	64	-
	Izeh, Esfahan Basin	33	67	-
	Dastjerd, Esfahan Basin	80	20	-
	Gavkhuni, Esfahan Basin	100	-	-
A. kavirensis	Damghan, Kavir Basin	100	-	-
A. mesopotamicus	Shadegan, Tigris Basin	71	29	-
A. pluristriatus	Khonj, Mond River Basin	50	40	10
A. shirini	Khosroshirin, Kor River Basin	62	38	-
A. sophiae	Ghadamgah, Kor River Subsystem	97	3	-
A. vladykovi	Chaghakhor, Tigris Basin	100	-	-
	Ghandoman, Tigris Basin	100	-	-
Genus: *Aphaniops*				
A. ginaonis	Genow, Hormuzgan Basin	18	-	82
A. stoliczkanus	5km Mirahmad, Helle Basin	-	-	100
	Ilam-Sartang, Tigris Basin	-	17	83
A. hormuzensis	Mehran-Rudan, Hormuzgan Basin	12.5	-	87.5
	Kahurestan, Hormuzgan Basin	33	-	67
	Mehran, Hormuzgan Basin	-	-	100
	Minab road, Hormuzgan Basin	-	14	86
	Khurgu, Hormuzgan Basin	-	-	100
	Mehran-Kukherd, Hormuzgan Basin	17	8	75
	Faryab, Hormuzgan Basin	33	-	67

Table 6. Percentage of different numbers of PU vertebrae and modified caudal vertebrae among the studied Aphaniid species.

Genus and Species	Region/Basis	Number of PU Vertebrae			Number of Modified Caudal Vertebrae		
Genus: *Aphanius*		2	3	4	0	1	2
A. arakensis	Arak, Namak Basin	60	40	-	45	55	-
	Karaj, Namak Basin	70	30	-	15	77	8
	Varamin, Namak Basin	43	57	-	14	85	-
A. darabensis	Darab, Hormuzgan Basin	100		-	-	100	-
A. farsicus	Babunak spring, Maharlu Lake System	86	14	-	21	68	11
A. isfahanensis	Varzaneh, Esfahan Basin	100		-	17	83	-
	Zayandehroud, Esfahan Basin	64	36	-	36	64	-
	Izeh, Esfahan Basin	100		-	16.7	66.7	16.7
	Gavkhuni, Esfahan Basin	50	50	-	50	50	-
	Dastjerd, Esfahan Basin	80	20	-	20	80	-
A. kavirensis	Damghan, Kavir Basin	67	33	-	33	67	-
A. mesopotamicus	Shadegan, Tigris Basin	50	50	-	12.5	87.5	-
A. pluristriatus	Khonj, Mond River Basin	70	30	-	40	60	-
A. shirini	Khosroshirin, Kor River Basin	44	56	-	89	11	-
A. sophiae	Ghadamgah, Kor River Subsystem	42	58	-	52	48	-
A. vladykovi	Chaghakhor, Tigris Basin	58	42	-	25	75	-
	Ghandoman, Tigris Basin	33	67	-	33	67	-
Genus: *Aphaniops*							
A. ginaonis	Genow, Hormuzgan Basin	-	82	18	9	82	9
A. stoliczkanus	5km Mirahmad, Helle Basin	-	100	-	-	100	-
	Ilam-Sartang, Tigris Basin	-	100	-	33	67	-
A. hormuzensis	Mehran-Rudan, Hormuzgan Basin	-	100	-	25	75	-
	Kahurestan, Hormuzgan Basin	-	100	-	-	100	-
	Mehran, Hormuzgan Basin	-	100	-	20	80	-
	Minab road, Hormuzgan Basin	14	86	-	14	86	-
	Khurgu, Hormuzgan Basin	-	100	-	60	40	-
	Mehran-Kukherd, Hormuzgan Basin	8	92	-	50	50	-
	Faryab, Hormuzgan Basin	-	100	-	33.3	33.3	33.3

In the case of *Aphaniops* species, no significant differences were detected among *Aphaniops* species in the number of modified caudal vertebrae (Kruskal-Wallis, $p>0.05$).

Neural and haemal spines width of PU vertebrae

The effect of the fish TL on the widths of the neural and haemal spine of PU2-4 was successfully eliminated using the residuals from the linear regression between these variables; the size-corrected variables showed no correlation ($r < 0.001$; $p > 0.05$). The neural and haemal spines of PU2-PU4 were significantly wider in the genus *Aphaniops* than in *Aphanius* (T-test, $p<0.05$ for all pairwise comparisons) (Figures 6, 7).

In the case of the genus *Aphanius*, in *A. arakensis*, the mean values of the neural spine of PU 2-4 width and hemal spine of PU2-3 width in Karaj and Varamin specimens were significantly higher than those of Arak locality (ANOVA, $p<0.05$).

Concerning the comparison of species, the value of the neural spine of PU2 in the specimens of *A. arakensis* from Varamin is significantly wider than other species (ANOVA, $p<0.05$), except for *A. kavirensis* and *A. pluristriatus* specimens. No significant differences were detected among species in the neural spine width of PU3 and PU4 (ANOVA, $p< 0.05$). Comparison of the value of haemal spine width of PU3 showed that the specimens of *A. arakensis* from Varamin were significantly larger than those of *A. shirini*, *A. sophiae,* and *A. vladykovi* (ANOVA, p< 0.05, for all pairwise comparison). No significant differences were detected among other species (ANOVA, p< 0.05, for all pairwise comparisons).

In the case of genus *Aphaniops*, the value of neural and haemal spine of PU2-PU4 in *A. ginaonis* were significantly different from those of *A. hormuzensis* (ANOVA, p< 0.05 for all pairwise comparisons).

Discussion

This study aimed to identify the diversity in the vertebral column and caudal skeleton traits in 10 *Aphanius* and three *Aphaniops* species. Ecomorphological theory indicates that body form evolves in response to the environmental challenges that are faced by living organisms (Wainwright and Reilly, 1994). The association between the body form and function is often strong in aquatic organisms, such as fish (Bidaye *et al*., 2022; Langerhans and Reznick, 2010; Webb, 1984).

In this study, we demonstrated that VBI as an effective parameter provided a reliable measurement to assess vertebral column curvature. How “almost straight” and “almost curved” should be defined as the variability in the curvature of the shape of the vertebral column, was not clear from previous studies. In the present study, the investigated specimens of the *Aphanius* and *Aphaniops* species showed three different shapes of the vertebral column based on the VBI index. Such intraspecific variations were not detected previously (Teimori and Esmaeili, 2020). Moreover, in the present study, no geographic pattern was detected in the shape of the vertebral column variation among the localities.

Change in the vertebral number is one of the most common patterns of divergence in body shape in fish associated with the important role of the vertebral column in bending the body during swimming (Brainerd and Patek, 1998; Nowroozi and Brainerd, 2012). Water flow and temperature which vary widely across ecosystems are important environmental factors that can influence the phenotypic characteristics of fishes (Angilletta, 2009). Temperature and water flow can also covary along the elevational gradients in streams. At higher elevations, streams are often colder and exhibit greater water velocity whereas, at lower elevations, they tend to be warmer and run more slowly as they broaden (Fowler, 1970; Jordan, 1891; Lindsey, 1975; Lindsey and Arnason, 1981; McDowall, 2008; Morris *et al*., 2017; Ward and Brainerd, 2007). Variation in temperature during development generally results in an inverse relationship between the vertebral number and temperature or a U-shaped pattern, with vertebral number being greater at temperature extremes and lower at intermediate temperatures (Fowler, 1970; Lindsey and Arnason, 1981). Previous studies have demonstrated that the effects of water flow on the body shape across taxonomically diverse groups, in which fish are exposed to a high-water flow rate, tend to be more streamlined to reduce the drag caused by the current (Sfakianakis *et al*., 2011; Langerhans and Reznick, 2010; Webb, 1984). Increases in the body elongation are strongly correlated with increases in the vertebral number. Additionally, the fish with more vertebrae have more streamlined bodies.

In this study, species with more total vertebrae are found in higher latitudes such as specimens of *A. arakensis*, and higher altitudes such as specimens of *A. shirini*, *A. sophiae,* and *A. vladykovi* than the remaining *Aphanius* species and the members of genus *Aphaniops*. Furthermore, in the study of Teimori and Esmaeili (2020), two species of the genus *Aphanius (*i.e., *A. anatoliae* from Anatolia and *A. arakensis* from the Namak Lake basin of Iran with 31 and 29 total vertebrae respectively) that are located at higher latitudes revealed the highest number of vertebrae. Moreover, *A. mesopotamicus* from the lower Tigris drainage from Iran and *A. asquamatus* from Anatolia with 26, showed the lowest total vertebrae. Although in the present study we do not have direct evidence, an increase in the number of vertebrae documented here may be related to lower water temperature or higher water flow of the streams at higher latitude or altitude that is relatively a common response to variation under different temperatures and water flow among fish. Some environmental factors possibly affect both the number of vertebrae and the shape of the vertebral column, and consequently, a specific combination may be chosen (Ackerly and Ward, 2016; Aguirre *et al*., 2019; Barriga *et al*., 2013; Sfakianakis *et al*., 2011).

The results of this study revealed that the number of principal caudal fin rays and segmented soft rays on the hypural plate was higher in *Aphanius* compared to *Aphaniops* species. *Aphaniops ginaonis* showed the lowest rays on the hypural plate. Moreover, the width of the neural and hemal spine of PU vertebrae was higher in *Aphaniops* compared to the *Aphanius* species. Consistent with this study, Teimori and Esmaeili (2020) showed that the number of rays on the hypural plate of the studied *Aphanius* species was 10-12 (often 11) higher than the genus *Aphaniops* 9-12 (10). Caudal fin musculature in teleost fish allowed precise control over their tail conformation (lauder & Drucker, 2004). The observed changes in the studied species in the number of principal caudal fin rays, rays on the hypural plate, and spine width of the PU vertebrae may cause different swimming activities and create different effective tail impacts during fish movement in an ecologically different habitat of the studied species.

In the specimens of the genus *Aphanius* and *Aphaniops*, two shapes of straight (two forms) and sinusoidal epural bone were found. Unlike *Aphanius*, in which two forms of straight epural bones were more frequent, *Aphaniops* showed more frequent sinusoidal epural bone in its skeleton. Such intra-species polymorphism of the epural characteristic state had not previously been reported for Aphaniid species. Charmpila *et al*. (2020) showed that epural bone in four *Aphaniops* species (*i.e*., *A. dispar*, *A. hormuzensis*, *A. kruppi*, and *A. stoliczkanus*) was all sinusoidal. Also, Teimori and Esmaeili (2020) reported that all species of the genus *Aphanius*, as well as *Paraphanius mento*, displayed a straight epural bone in their caudal skeleton, while all species of the genus *Aphaniops* (except *A. furcatus*) exhibited a sinusoidal shape. In sum, these studies indicated that the sinusoidal shape of the epural bone could be a synapomorphy for the “inner” clade of *Aphaniops* species. Examining more specimens of all *Aphanius* species in the present study revealed the intraspecies polymorphisms of the epural character state. These findings are consistent with the report of the sinusoidal epural bone by Costa (2012) for *Aphanius isfahanensis*. The previously suggested synapomorphies for Aphaniid species based on the shape of the epural bone of *Aphanius* and *Aphaniops* species may need further examination.

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Conflicts of interest

The authors have declared no conflicts of interest.

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