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Research Article

Taxonomic status of the genus *Cobitis* (Teleostei: Cobitidae) in the Namak Lake basin, Iran

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Abstract: This study was aimed to report the presence of the genus *Cobitis* in the Karaj River, Namak Lake basin, Iran and clarify its taxonomic status by providing morphological characteristics, mtDNA COI barcode region and its phylogenetic relationship within the members of the genus *Cobitis* in Iran. The results revealed that morphometric, meristic and molecular (COI) characters of these specimen are largely overlapping or even identical with those of *C. faridpaki*. Therefore, we conclude that they are a population belonging to *C. faridpaki*.

Keywords: Taxonomy, Morphology, COI, Spined loach, Distribution.

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Introduction

The members of the genus *Cobitis* Linnaeus, 1758 represent one of the most widely distributed Palearctic primary freshwater fishes (Sawada 1982; Coad 2017). They are found in Eurasia and Morocco (North Africa) and Southern Asia (Eschmeyer & Fong 2011). This genus has four valid species in Iran (Mousavi-Sabet et al. 2011; Joulade-Roudbar et al. 2017; Eagderi et al. 2017). *Cobitis linea* Heckel, 1849 is found in the Kor River and upper Kol River drainages, *C. avicennae* Mousavi-Sabet, Vatandoust, Esmaeili, Geiger & Freyhof, 2015 occurs in the Karkheh and Karun, two sub-tributaries of the Tigris, *C. faridpaki* Mousavi-Sabet, Vasil'eva, Vatandoust & Vasil'ev, 2011 was described from the Siah River (eastern part of the Iranian Caspian Sea basin) and *C. saniae*, Eagderi, Jouladeh-Roudbar, Jalili, Sayyadzadeh & Esmaeili, 2017 was recently described from the Sefid River (western part of the Iranian Caspian Sea basin) (Mousavi-Sabet et al. 2011, 2015; Jouladeh-Roudbar et al. 2015, Eagderi et al. 2017; Esmaeili et al. 2017).

We collected spined loaches of the genus *Cobitis* from the Karaj River drainage (Namak Lake basin, Iran) in 2016. Therefore, this study was aimed to report presence of the genus *Cobitis* in the Namak Lake basin and clarify its taxonomic status by providing morphological characteristics, mtDNA COI barcode region and its phylogenetic relationship within the members of the genus *Cobitis* in Iran.

Materials and Methods

Twelve specimens of spined loach were collected from the Karaj River, Namak Lake basin, Iran

Table 1. Morphometric data of *Cobitis faridpaki* from the Namak Lake and Caspian Sea basins.

Characters	Siah River				Karaj River			
	male (n=7)		female (n=12)		male (n=6)		female (n=5)	
	range	mean±SD	range	mean±SD	mange	mean±SD	range	mean±SD
Standard length (mm)	49.2-61.6		37.0-68.0		61.8-92.1		57.8-68.4	
In percent of standard length								
Body depth at dorsal fin origin	16.1-18.3	16.6±0.8	15.3-17.9	16.3±0.8	15.7-17.9	16.9±0.8	15.7-19	17.0±1.2
Caudal peduncle depth	10.1-12.9	11.2±1.0	9.4-11.3	10.4±0.6	8.5-10.3	9.5±0.6	8.7-11.5	9.9±1.0
Predorsal length	50.8-54.9	52.5±1.6	50.8-53.9	52.4±1.1	53.2-55.8	54.2±0.8	53.9-57.6	55.5±1.3
Postdorsal length	44.7-48.6	47.1±1.2	42.4-49.7	46.4±2.5	45.1-49.6	47.5±1.5	44.5-47.8	46.5±1.2
Prepelvic length	56.7-57.8	57.3±0.5	51.6-58.1	56.0±1.7	52.3-57.4	53.8±1.6	54-57.9	55.4±1.5
Preanal length	80.4-83.0	81.6±0.9	79.6-83.0	81.3±1.1	78.7-82.7	80.8±1.4	78.9-82.8	81.4±1.4
Caudal peduncle length	11.2-13.8	12.8±0.8	11.1-13.9	12.6±0.9	11.4-15.7	14.1±1.4	12.5-15.5	13.5±1
Dorsal-fin base length	7.3-11.1	9.4±1.4	7.6-12.6	9.4±1.4	6.7-9.9	8.0±1.0	7.3-9.9	8.2±0.8
Dorsal-fin depth	10.5-17.8	14.2±2.6	12.8-17.6	14.4±1.6	11.6-14.4	13.1±1.1	11.7-16.1	13.8±1.5
Anal-fin base length	6.3-8.4	7.3±0.7	4.4-7.7	6.5±1.0	4.9-7.6	6.1±1.0	5.2-7.2	6.2±0.7
Anal-fin depth	11.0-13.9	12.7±1.1	8.8-12.8	11.4±1.2	6.9-10.7	8.9±1.4	7.7-10.3	9.1±0.9
Pectoral fin length	14.9-18.5	17.2±1.5	13.0-18.6	15.2±1.7	9-13.3	11.3±1.3	9-13.3	11.9±1.5
Pelvic fin length	10.6-14.5	12.3±1.3	9.8-14.5	12.1±1.4	10.3-12.6	11.3±0.8	8.9-12.3	10.8±1.3
Distance between pectoral and pelvic-fin origins	33.4-38.2	35.4±1.6	30.8-36.8	34.1±1.9	31.3-35.8	34±1.4	31.0-37.0	34.7±1.8
Distance between pelvic and anal-fin origins	23.8-25.9	25.1±0.8	23.9-29	26.1±1.4	25.7-29.8	27.5±1.2	24.8-27.8	26.2±1.1
Body width at dorsal fin origin	9.4-11.9	10.7±0.8	9.3-12.5	10.2±0.9	9.3-11	10±0.9	9.1–10.8	10±0.7
Caudal peduncle width	2.7-3.2	2.9±0.2	2.3-3.7	2.9±0.4	1.5-2.2	1.8±0.3	1.4-2.1	1.6±0.4
Head length (HL)	19.2-21.4	20.2±0.9	17.5-21.9	20.4±1.3	16.0-20.2	17.8±1.7	16.6-19.2	18±1.0
In percent of head length								
Snout length	40.8-43.4	41.7±0.8	37.0-47.1	40.6±3.1	41.1-52.3	46.8±4.2	40.8-50.2	46.5±2.9
Horizontal eye diameter	14.9-20.2	17.8±1.7	15.9-20.9	18.3±1.8	11.5-16.3	14±1.5	12.7-18.8	14.3±2.1
Postorbital distance	44.0-59.2	50.1±4.8	47.1-61.9	51.4±4.1	48.8-59.6	55.2±3.4	50-62.1	55.8±3.7
Head depth at nape	65.3-75.6	70.3±3.6	65.3-80.2	69.2±4.1	64.6-77.9	72.4±4.5	67.8-77.6	72.8±3.6
Head depth at eye	53.4-61.3	58.5±2.4	53.6-64.5	58.0±3.4	53.4-69.1	60.1±4.3	55.7-67.1	61.8±3.9
Dorsal head length	84.0-90.1	87.6±2.2	83.5-91.1	87.3±2.4	84.7-90.7	87.0±3.3	82.0-89.6	85.1±4.9
Head width at nape	37.3-52.5	44.1±4.7	38.8-53.0	44.1±4.6	49.9-52.2	51.4±1.3	48.1-50.3	52.0±2.1
Interorbital distance	14.8-18.5	16.8±1.3	12.9-20.5	16.3±2.2	20.6-25.3	23.6±2.6	19.2-22.1	21.1±1.3
Internasal distance	13.4-22.5	19.4±2.8	16.2-23.7	19.8±2.5	15.6-18.5	17.2±1.5	16.4-17.2	16.3±1.1
Mouth width	13.2-17.5	15.9±1.7	12.4-19.4	15.7±2.1	13.7-17.0	15.4±1.7	12.5-16.1	15.1±1.3
Inner rostral barbel length	12.2-16.6	13.3±2.9	5.9-16.0	10.6±2.8	13.3-21.1	16.5±4.1	12.2-20.2	15.5±6.1
Outer rostral barbel length	13.4-20.6	17.1±2.4	8.0-18.2	15.0±2.9	15.4-17.7	16.4±1.2	15.6-16.1	15.1±1.0
Maxillary barbel length	19.3-27.1	20.9±4.9	14.1-23.5	19.4±3.6	13.0-22.1	17.4±4.6	13.5-22.9	18.4±3.2

(Alborz Province) in May 2016 with an electrofishing device. After anesthesia, three specimens were fixed in 96% ethanol for molecular studies and the other specimens were preserved in 5% buffered formaldehyde.

Morphological analysis: A total of 32 morphometric features were measured by a digital caliper to the nearest 0.01mm (Table 1). All measurements are

Table 2. Meristic data of *Cobitis faridpaki* from Siah and Karaj Rivers.

Characters	Siah River				Karaj River			
	male (n=7)		female (n=12)		male (n=6)		female (n=5)	
	range	mode	range	mode	range	mode	range	mode
Blotches in Z4	14-20	17	14-20	19	17-22	17	15-25	17
Predorsal blotches in Z4	7-12	12	6-15	7-8	11-18	15	9-16	12
Postdorsal blotches in Z4	6-11	8	5-15	7	12-16	13	8-15	11
Branched dorsal-fin rays	6-7	7	6-8	7	7-8	7	7-8	7
Branched anal-fin rays	5-6	6	5-6	6	6-6	6	5-6	6
Pectoral-fin rays	7-8	7	6-8	7	7-7	7	6-7	7
Pelvic-fin rays	6-7	6	6-7	6	6-7	6	6-7	7
Caudal-fin rays	16-17	16	16-18	16	16-17	16	16-17	16

made point to point based on Kottelat & Freyhof (2007). Standard length (SL) is measured from the tip of the snout to the end of the hypural complex. The length of the caudal peduncle is measured from behind the base of the last anal-fin ray to the end of the hypural complex, at mid-height of the caudal-fin base. The last two branched rays articulating on a single pterygiophore in the dorsal and anal fins are counted as "1½". The percentage ratios of morphometric characters in relations to SL and HL were calculated. Eight meristic characteristics of the specimens were counted using a stereomicroscope (Table 2). Terminology of the pigmentation pattern follows Kottelat & Freyhof (2007).

DNA extraction and PCR: DNA was extracted from the collected muscle tissues using a Genomic DNA Purification Kit (#K0512; Thermo Scientific Corporation, Lithuania) following the manufacturer's protocol. The COI gene was amplified using primers FCOI20-(5'- AACCTCTGTCTTCGGGGCTA -3') and RCOI20III-(5'- TTGAGCCTCCGTGAAGTG T G- 3') (Hashemzadeh Segherloo et al. 2012). Polymerase chain reaction (PCR) conditions were as follows: a 50µl final reaction volume containing 5µl of 10X Taq polymerase buffer, 1µl of (50 mM) $MgCl_2$, 1µl of (10mM) deoxynucleotide triphosphate (dNTP), 1µl (10µm) of each primer, 1µl of Taq polymerase ($5U\mu l^{-1}$), 7µl of total DNA and 34µl of H_2O. Amplification cycles were as follows: denaturation for 10 min at 94°C; 30 cycles at 94°C for 1min, 58.5°C for 1min, 72°C for 1min and a final extension for 5min at 72°C. PCR products were purified using purification Kit (Expin Combo GP – mini; Macrogen incorporation, Korea). The PCR products were sequenced using Sanger method by a robotic ABI-3130xl sequencer using manufacturer's protocol. The forward and reverse primers were used to single strand sequencing.

Molecular data analysis: The sequences were compared to published *Cobitis* sequences using (BLASTn) basic local alignment search tool (Altschul et al. 1990). The retrieved sequences of the other members of the genus *Cobitis* from GenBank database (NCBI) following blast search are shown in Table 3. Sequence data were aligned using MEGA7 software (Tamura et al. 2013). Sequences of COI gene were trimmed to the size of the smallest fragment, resulting in a dataset of 644 base pairs (bp). Modeltest (Posada & Crandall 1998), implemented in the MEGA7 software (Tamura et al. 2013) was used to determine the most appropriate sequence evolution model for the given data, treating gaps and missing data with the partial deletion option under 95% site coverage cut-off. The model with the lowest BIC scores (Bayesian Information Criterion) is considered to best describe the substitution pattern (Nei & Kumar 2000; Posada & Crandall 2001). Bayesian analyses of nucleotide sequences were run with the parallel version of MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) on a Linux cluster with one

Fig.1. Lateral view of *Cobitis faridpaki*, (above) from the Karaj River, Namak Lake basin, and (below) from the Siahrud River, Mazandran prov., Iran.

processor assigned to each Markov chain under the most generalizing model (HKY+G) because over parametrization apparently does not negatively affect Bayesian analyses (Huelsenbeck & Ranala 2004). Two simultaneous analyses were run on $1.5x10^7$ generations, each with four MCMC chains sampling every 100 generations. Convergence was checked on Tracer 1.6 (Rambaut & Drummond 2013). Analyses were terminated after the chains converged significantly, as indicated by the average standard deviation of split frequencies <0.01. Estimates of evolutionary divergence over sequence pairs between species were conducted in Mega7 (Tamura et al. 2013). Analyses were conducted using the Kimura 2-parameter model (Kimura 1980). The rate variation among sites was modelled with a gamma distribution (shape parameter=1). Codon positions included were 1st+2nd+3rd. All positions containing gaps and missing data were eliminated. As outgroup, three *Misgurnus fossilis* samples were retrieved from GenBank database (accession numbers: KM286763-5).

Abbreviations used: SL, standard length; HL, lateral head length; Z4, midlateral row of dark-brown blotches along the flank, K2P, Kimura 2-parameter; IMNRFI-UT, Ichthyological Museum of Natural Resources Faculty, University of Tehran. ZM-CBSU, Zoological Museum of Shiraz University, Collection of Biology Department, Shiraz.

Results

Morphological analysis: The general body shape is displayed in Figure 1 and the morphological characteristics are provided in Tables 1-2. Based on the results, the morphometric and meristic characters, and colour patterns i.e. Gambetta's zones of pigmentations of the collected specimens from the Karaj River show similar variations with those of *C. faridpaki* from its type locality i.e. Siah River, Caspian Sea basin (Tables 1-2). Morphometric characters of *C. faridpaki* from the Namak and Caspian Sea basins overlap except the caudal peduncle width that is shorter (1.4-2.2 vs. 2.3-3.7) in those of the Karaj River.

Table 3. List of species used for molecular analysis for COI and GenBank Accession Number.

No.	Accession no.	Species	No.	Accession no.	Species
1	KP050508	*Cobitis avicennae*	24	HQ536325	*Cobitis lutheri*
2	KP050525	*Cobitis avicennae*	25	HQ536326	*Cobitis lutheri*
3	KP050516	*Cobitis avicennae*	26	KP050528	*Cobitis saniae*
4	KJ552817	*Cobitis battalgili*	27	KP050506	*Cobitis saniae*
5	KJ552834	*Cobitis battalgili*	28	KY646319	*Cobitis saniae*
6	KJ552796	*Cobitis battalgili*	29	KY646320	*Cobitis saniae*
7	KJ553211	*Cobitis bilineata*	30	KY646321	*Cobitis saniae*
8	KJ552762	*Cobitis bilineata*	31	KY646322	*Cobitis saniae*
9	KJ553176	*Cobitis bilineata*	32	KP050509	*Cobitis saniae*
10	KP050514	*Cobitis elazigensis*	33	KP050518	*Cobitis saniae*
11	KP050527	*Cobitis elazigensis*	34	KJ128460	*Cobitis taenia*
12	KP050513	*Cobitis elazigensis*	35	KJ128459	*Cobitis taenia*
13	KY476338	*Cobitis faridpaki*	36	KM286524	*Cobitis taenia*
14	KY476339	*Cobitis faridpaki*	37	KJ553220	*Cobitis turcica*
15	KY476334	*Cobitis faridpaki*	38	KJ552782	*Cobitis turcica*
16	KY476337	*Cobitis faridpaki*	39	KJ552985	*Cobitis turcica*
17	KY476336	*Cobitis faridpaki*	40	KJ553193	*Cobitis zanandreai*
18	KY646316	*Cobitis faridpaki*	41	KJ553015	*Cobitis zanandreai*
19	KY646317	*Cobitis faridpaki*	42	KJ553001	*Cobitis zanandreai*
20	KY646318	*Cobitis faridpaki*	43	KM286764	*Misgurnus fossilis*
21	KP050530	*Cobitis linea*	44	KM286763	*Misgurnus fossilis*
22	KP050539	*Cobitis linea*	45	KM286765	*Misgurnus fossilis*
23	HQ536324	*Cobitis lutheri*			

Table 4. Estimates of the average evolutionary divergence between the analysed *Cobitis* species. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated.

Species	No.	1	2	3	4	5	6	7	8	9	10	11
C. avicennae	1											
C. battalgili	2	8.41										
C. bilineata	3	10.97	10.97									
C. elazigensis	4	7.98	7.06	9.00								
C. faridpaki (Caspian basin)	5	5.25	9.06	11.52	7.89							
C. faridpaki (Namak basin)	6	5.23	8.98	11.67	8.08	0.29						
C. linea	7	10.97	8.01	9.95	4.48	10.36	10.33					
C. lutheri	8	10.46	9.86	9.82	8.50	10.96	11.13	9.63				
C. saniae	9	5.29	9.00	11.44	8.65	2.33	2.23	10.69	10.89			
C. taenia	10	4.83	7.65	11.14	7.54	5.95	5.87	9.43	10.34	5.58		
C. turcica	11	8.31	1.14	10.53	7.28	8.90	8.88	8.26	10.08	9.23	8.18	
C. zanandreai	12	12.93	11.95	9.00	10.15	12.37	12.49	10.95	10.26	12.71	12.45	11.52

Molecular analysis: We are able to generate three new COI sequences (see below). Additional 42 sequences of 12 species were downloaded from Genbank (Table 3). Two phylogenetic approaches Bayesian Inference (BI) and Maximum Likelihood (ML), gave the same tree topologies and thus one is presented (Fig. 2).

Table 4 lists estimates of the average evolutionary divergence found in the mtDNA COI barcode region. According to the results, *Cobitis faridpaki* from the

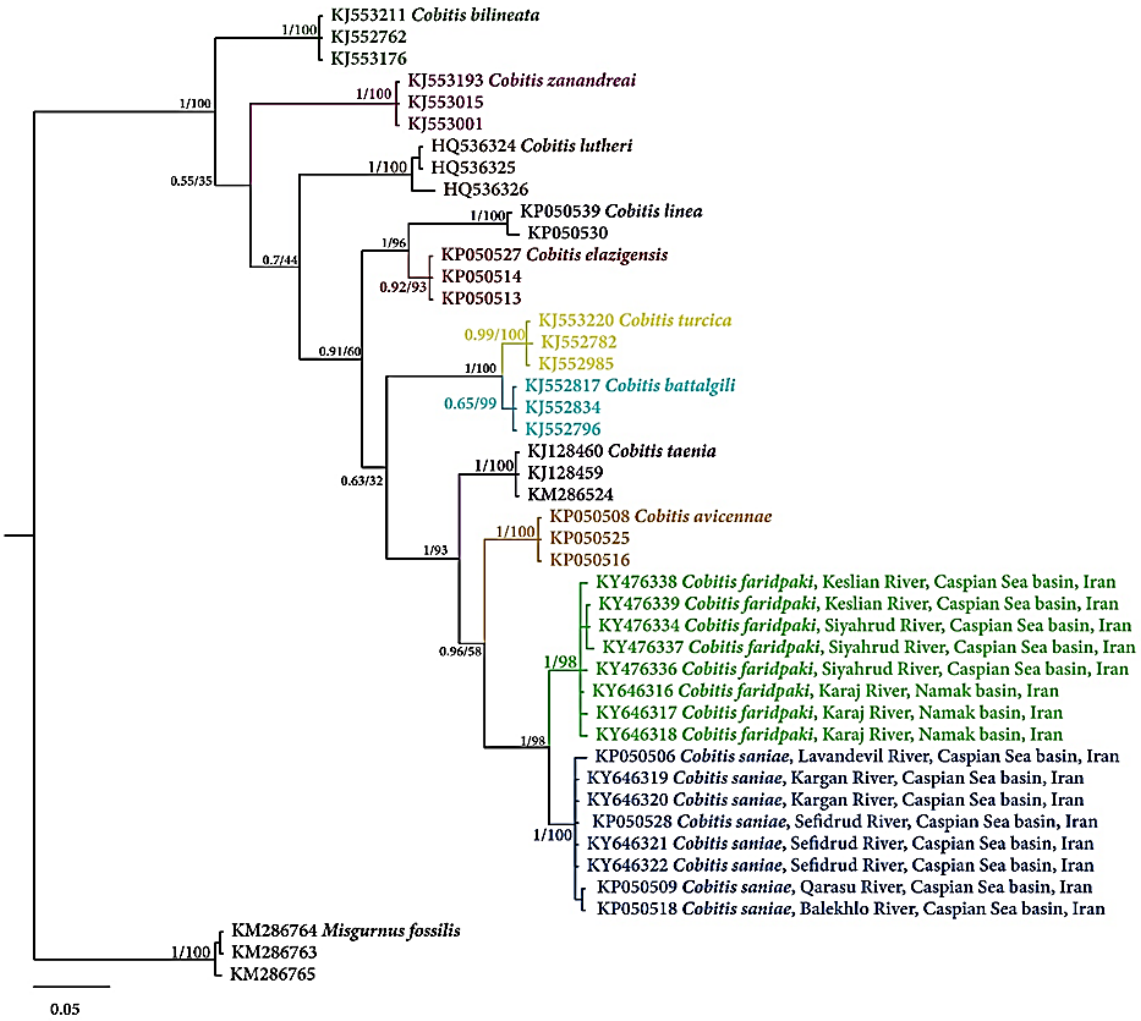

Fig.2. Bayesian consensus tree inferred from COI data. Values at nodes correspond to BI posterior probability/ML bootstrap. Numbers before each species correspond to the GenBank accession number.

Karaj River was nested within species of *C. faridpaki* and they are indistinguishable in their diagnostic nucleotide substitutions. In addition, the genetic distance between *Cobitis faridpaki* from the Karaj River and Siah River (based on specimens from its type localities) was 0.29%.

Discussion

The family Cobitidae, spined loaches, has 21 genera with about 195 species (Nelson et al. 2016). The genus *Cobitis* is complicated and its systematics is poorly recognized (Bohlen & Ráb 2001). The members of this genus show a great variations in terms of morphological and colour patterns. Therefore, describing new species of the genus *Cobitis* solely based on morphological and colour patterns can be lead to erroneous new species names being given to valid species (Carlton 2007) as seen in the case of *C. keyvani* (Jouladeh-Roudbar et al. 2017). Hence, application of DNA markers along with traditional morphological characters are crucial to confirm the species true taxonomic status particularly in the case of the genus *Cobitis*.

The results of the present study revealed that almost all of the morphometric and meristic characters of *Cobitis* specimens from the Karaj River are largely overlapping with those of *C. faridpaki* from the Siah River, the Caspian Sea basin. However, there is difference in the caudal peduncle width between the two populations, which could be a result

of phenotypic plasticity due to different ecological conditions of their habitats (Nakamura 2003).

Based on the results, specimens of the genus *Cobitis* from the Karaj River were placed within same clade with *C. faridpaki* from the Caspian Sea basin with 0.29% genetic distance. The genetic distance between well-known *Cobitis* species is minimum 1.14% far from the 0.29% observed between populations of *C. faridpaki* from the Karaj and Siah rivers (Table 4) i.e. the pairwise distance of these two populations is a normal range of divergence between populations of a single species. In addition, the genetic distance between populations of *C. faridpaki* in the Caspian Sea basin was reported to be 0.12–0.17% (Jouladeh-Roudbar et al. 2017).

According to morphological and molecular data, it is evidence from the absence of any diagnostic morphological character and minor differences in the COI sequences (0.29%), we conclude that the specimens of *Cobitis* from the Karaj River are a population belonging to *C. faridpaki.*

Materials used for Morphological analyses: All from Iran: *Cobitis faridpaki*: — IMNRF-UT-1016, 19, 37-68mm SL; Mazandaran prov.: Siah River at Ghaemshahr, Caspian Sea basin, 36°26'39.0"N 52°53'43.6"E; S. Eagderi & A. Jouladeh-Roudbar, Aug 2016. — IMNRF-UT-1015, 21, 51-90mm SL; Mazandaran prov.: Keselian River at Savadkoh, Caspian Sea basin, 36°12'19.1"N53°00'56.0"E; S. Eagderi & A. Jouladeh-Roudbar, July 2015. IMNRF-UT-1100, 11, 37-68mm SL; Alborz prov.: Karaj River at Asara, Namak Lake basin, 36°1'52"N 51°12'51"E, S. Eagderi & M. Nasri, May 2016. — ZM-CBSU H2007, 20, 42-67mm SL; Siah River, Ghaemshahr at Saru Kola village, 36°27'26.50"N 52°53'28.75"E, H.R. Esmaeili, 2014.

Cobitis saniae: — IMNRF-UT-1018, 6, 38-53mm SL; Gilan prov.: Sefid River at Totkaboon, Caspian Sea basin, 36°53'27.3"N 49°30'42.0"E; S. Eagderi, July 2014.

Cobitis avicennae: — IMNRF-UT-1096, 12, 71-115mm SL; Kermanshah prov., Dinevar River at Hossein Abad, Tigris drainage, 34°33'16.6"N 47°24'48.4"E; A. Soleymani, T. Hossein pour & A. Jouladeh-Roudbar, Aug 2016. — IMNRF-UT-1020, 1, 95mm SL; Kermanshah prov.; Dinevar River at Hossein Abad, Tigris drainage, 34°33'16.6"N 47°24'48.4"E; S. Eagderi & A. Jouladeh-Roudbar, Jun 2016.

Cobitis linea: — ZM-CBSU H2090, 6, 53-79mm SL; Fars prov.: Ghadamgah spring at Dorudzan, Kor river basin, 30°14'19.65"N 52°22'23.3"E; G. Sayyadzadeh, S. Mirghiasi & S. Ghasemian, May 2013. — ZM-CBSU H2096, 6, 45-72mm SL; Fars prov.: Ghadamgah spring at Dorudzan, Kor river basin, 30°14'19.65"N 52°22'23.3"E; H.R. Esmaeili, V. Niknejad & Ebrahimi, August 2004.

Materials used for molecular analyses: *Cobitis faridpaki*: IMNRF-UT-1100-1-fin clip; Alborz prov.: Karaj River at Asara, Namak Lake basin, 36°1'52"N 51°12'51"E, S. Eagderi & M. Nasri, May 2016, GeneBank Accession number (KY646316, KY646317, KY646318).

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مقاله پژوهشی

وضعیت آرایه‌شناختی جنس *Cobitis* (ماهیان استخوانی عالی: سگ‌ماهیان جویباری خاردار) حوضه دریاچه نمک ایران

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چکیده: هدف این مطالعه گزارش حضور جنس *Cobitis* در رودخانه کرج، حوضه دریاچه نمک، ایران و مشخص کردن وضعیت آرایه-شناختی آن با فراهم کردن داده‌های ریخت‌سنجی، بارکد ژن سیتوکروم اکسیداز میتوکندریایی و رابطه تبارشناختی آن با دیگر اعضای جنس *Cobitis* در ایران است. نتایج نشان داد که ویژگی‌های ریخت‌سنجی اندازشی و شمارشی و مولکولی نمونه‌های گردآوری شده با نمونه های گونه سگ‌ماهی خاردار فریدپاکی *C. faridpaki* مطابقت دارند. بنابراین نتیجه‌گیری شد که نمونه‌های جنس *Cobitis* رودخانه کرج یک جمعیت متعلق به سگ‌ماهی خاردار فریدپاکی می‌باشد.

کلمات‌کلیدی: تاکسونومی، ریخت‌شناسی، ژن سیتوکروم اکسیداز میتوکندریایی، سگ ماهی جویباری خاردار، پراکنش.