Research Article

A note on the molecular systematics of the genus *Paraschistura* (Teleostei: Nemacheilidae) in Hari River Basin

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Abstract: Nemacheilid loaches of the genus *Paraschistura* has been widely distributed from the Tigris drainage in Turkey, east throughout Iran and Pakistan to the Indus River and the Hari, Murghab and Helmand endorheic basins in Afghanistan, Iran, Pakistan and Turkmenistan. Herein, *Paraschistura* specimens from seven stations in Hari river basin were captured using hand-net and COI sequences of *Paraschistura cristata* and *Paraschistura turcmenica* were presented for all recognized species. Moreover; 119 sequences from ten species of *Paraschistura* in Genbank were added in order to assess of the phylogenetic position of the *Paraschistura* specimens and compare resulting tree with other studies. Molecular data obtained from Maximum likelihood (ML) tree and Bayesian tree (BI) were congruent and clearly indicate two main clades in phylogenetic tree and the genus *Paraschistura* in the Hari River basin, are considered as *P. cristata* and *P. turcmenica*.

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Introduction
The members of the family Nemacheilidae, with about 70 genera and more than 668 valid species, are found across Eurasia with one species in northeast Africa (Eschmeyer & Fong 2016). This family has a great diversity in Iranian interior waters (Coad 2016) and some of its members have been the subject of ichthyological studies (Azimi et al. 2015a, b; Freyhof etal. 2015; Jamali et al. 2014,2015,2016; Mousavi-Sabet & Eagderi 2015; Sayyadzadeh et al. 2016). Classification of these taxa are complex and researches are trying to determine their phylogenic status (Prokofiev 2010). Recent classifications of members of the family Nemacheilidae have been merely based on external morphology and to a small extent on anatomy, osteology and molecular data (Bănărescu & Nalbant

1995; Freyhof et al. 2015; Sayyadzadeh et al. 2016).

Among the members of nemacheilids, the genus *Paraschistura* Prokofiev, 2009 was known to be a group of poorly known species especially in Iran. However, Freyhof et al. (2015) reviewed the Iranian species of *Paraschistura* and provided the diagnostic characters and molecular systematics for all eleven recognized species. Mousavi-Sabet et al. (2015) also reviewed the genus *Paraschistura* in the Hari River basin and presented diagnostic characters for all the three recognized species. Members of this genus with a dark black spot or strip at the base of the anterior dorsal fin rays has been widely distributed from the upper Tigris River basin, interior water bodies of Turkmenistan and from Iranian Baluchistan east to the upper

reaches of the Indus River in Afghanistan and Pakistan (Prokofiev 2009; Coad 2016). There are 29 valid species for genus *Paraschistura* based on Eschmeyer & Fong (2016).

Kottelat (2012) introduced 14 valid species were belonged to the genus *Paraschistura*, which are described from Afghanistan (*P. lindbergi*), Pakistan (*P. alepidota*, *P. kessleri*, *P. lepidocaulis*, *P. microlabra*, *P. naseeri*, *P. pakistanica*, *P. prashari*, *P. punjabensis*), Turkey (*P. chrysicristinae*), and Turkmenistan (*P. turcomana*), and the three remain species are endemic to Iran, including *P. bampurensis*, *P. nielseni* and *P. sargadensis*.

The *Paraschistura* genus in Iran was reviewed by Freyhof et al. (2015) and they described six new species including; *P. abdolii*, *P. aredvii*, *P. hormuzensis*, *P. naumanni*, *P. pasatigris* and *P. susiani*. Also they suggested that *P. sargadensis* is a synonym of *P. kessleri*, and *P. turcomana* is a synonym of *P. turcmenica*. Prokofiev (2009) established the genus *Metaschistura*, for *Nemachilus cristatus*; however, this species is now recognized as *Paraschistura cristata* by Freyhof et al. (2015). Mousavi-Sabet & Eagderi (2015) described another new species from the Mond River drainage (Persis sub-basin) in the Persian Gulf basin based on morphological characters and stated that this new species (*Paraschistura delvarii*) corresponds the unnamed sequences in phylogenetic tree of Freyhof et al. (2015). *Paraschistura alta* has been recorded and characterized from Zahak, Southeast of Iran for the first time based on the morphological and molecular data (Jouladeh-Roudbar et al. 2015). Until now, there are 12 valid species of *Paraschistura* in Iran (Jouladeh-Roudbar et al. 2015). *Paraschistura cristata* and *Paraschistura turcmenica* are recorded for the first time from Khorasan-Razavi based on only morphological characters (Yazdani-Moghaddam et al. 2015). *Paraschistura turcomana* is a poorly known species from the Kushk River in the Murghab drainage at the border of Afghanistan and Turkmenistan. Its validity has been questioned and a synonymy with *P. turcmenica* has been suggested by Freyhof et al. (2015), although Mousavi-Sabet et al. (2015) considered it as valid species.

The morphological and molecular comparisons of *Paraschistura* specimens from the Hari River in the northeastern Iran, with their nominal congeners in Iranian river basins, demonstrates that they clearly represent two valid Species of *Paraschistura*, which are described here. The aim of this study is to determine; (1) the current taxonomic status of the genus *Paraschistura* in the Hari River basin based on morphology and molecular examinations (2) compare the phylogenetic tree based on mtDNA COI barcode region with other molecular studies.

Materials and Methods

Sample collection and measurements. A total of 165 *Paraschistura* specimens were collected by hand-net and electrofishing throughout Hari River during 2010-2014 (Table 1). Hari River (Tedzhen) is an endorheic basin which originates from the Hindu Kush Mountains in Afghanistan, passes from northeastern Iran and ends in the Karakum desert in Turkmenistan (Fig. 1, Table 1). Collected specimens were fixed in 10% formaldehyde and stored in 70 % ethanol or directly fixed in 99 % ethanol. Morphometric characters were measured by a dial caliper to the nearest 0.1 mm. All measurements are made point to point, never by projections. Methods for counts and morphometric measurements were performed based on Kottelat & Freyhof (2007) and Mousavi-Sabet et al. (2015).

The voucher specimens are deposited in the Zoological Museum of the Ferdowsi University of Mashhad (ZMFUM). Specimens were identified according to Freyhof et al. (2015) identification keys.

Eight meristic characters were recorded including, dorsal fin unbranched rays (DFUR), dorsal fin branched rays (DFBR), pectoral fin unbranched rays (PFUR), pectoral fin branched rays (PFBR), ventral fin rays (VFR), anal fin unbranched rays (AFUR), anal fin branched rays (AFBR), lateral line scales (LLS) (Table 2). Also twenty conventional morphometric characters were measured for individual specimens, using digital Vernier calipers. Measurements included total length (TL), standard length (SL), head length (HL), head depth (HD),

head width (HW), eye diameter (ED), interorbital length (IL), preorbital length (PrO), postorbital length (PoO), body depth (BD), length of dorsal fin base (LDFB), dorsal-fin length (DFL), length of anal fin base (LAFB), anal-fin length (AFL), length of pelvic fin base (LPlFB), length of pectoral fin base (LPcFB), caudal-peduncle depth (CPD), predorsal distance (PDD), preventral distance (PVD), preanal distance (PAD) (Table 3).

Eight meristic characters and nineteen morphometric (as a ratio of total length) characters were used to conduct a Principal Component Analysis (PCA) between *P. cristata* and *P. turcmenica*, using PAST v. 2.06 (Hammer et al. 2001).

Table 1. Number of sequenced specimens, Location and coordinate of each species.

Location	Species	Morphologica	Molecular	Coordinate
Pole-Khatun	*Paraschistura cristata*	-	2	35°57´N 61°07´E
Darungar	*Paraschistura cristata*	-	2	37° 36´N58°38´E
Radkan	*Paraschistura cristata*	10	2	36°52´N 59°01´E
Abghad	*Paraschistura cristata*	-	2	36°29´N 59°02´E
Baghe-Khan	*Paraschistura turcmenica*	-	1	36°17´N 60°25´E
Kleilagh	*Paraschistura turcmenica*	10	2	35° 31´N59°53´E
Sarasiab	*Paraschistura turcmenica*	-	2	36°17'N 60°35´E

Table 2. Twenty morphometric characters of *Paraschistura cristata* and *Paraschistura turcmenica* measured in this study, including total length (TL), standard length (SL), head length (HL), head depth (HD), head width (HW), eye diameter (ED), interorbital length (IL), preorbital length (PrO), postorbital length (PoO), body depth (BD), length of dorsal fin base (LDFB), dorsal-fin length (DFL), length of anal fin base (LAFB), anal-fin length (AFL), length of pelvic fin base (LPlFB), length of pectoral fin base (LPcFB), caudal-peduncle depth (CPD), predorsal distance (PDD), preventral distance (PVD), preanal distance (PAD).

Measurements	*P. cristata*				*P. turcmenica*			
	Min	Max	Mean	Sd	Min	Max	Mean	Std.
TL	58.60	84.30	71.62	8.84895	37.19	49.23	44.5590	4.25797
SL	52.30	71.00	59.27	6.97855	30.29	40.53	35.7080	3.85060
HL	11.80	15.90	13.51	1.30848	6.91	8.81	7.9850	0.65405
HD	7.50	10.20	8.40	1.03064	3.97	4.76	4.4130	0.21396
HW	9.00	11.60	10.01	1.06087	4.20	5.97	4.9590	0.63466
PrO	5.40	8.00	6.53	0.82872	2.78	3.83	3.1750	0.37005
PoO	4.20	7.20	5.64	1.05746	3.04	4.01	3.5680	0.37835
IL	2.80	4.00	3.35	0.38944	2.02	2.80	2.3790	0.29327
ED	2.00	2.40	2.24	0.12649	1.10	1.57	1.3780	0.15605
PDD	25.90	36.70	31.06	4.02056	16.57	21.19	19.3390	1.64995
LDFB	6.80	10.50	8.41	1.33621	3.12	5.03	4.1760	0.63820
DFL	8.20	12.20	9.95	1.36158	5.88	8.61	7.2500	0.83319
LAFB	3.20	6.30	4.58	1.02935	2.18	3.66	2.8490	0.47962
AFL	7.00	11.10	8.41	1.09995	5.17	7.21	6.0480	0.79477
PAD	38.10	56.30	46.77	6.50983	22.07	30.23	27.3610	2.82966
LPcFB	2.10	3.20	2.71	0.44083	1.28	3.11	1.9930	0.63124
LPlFB	2.10	3.10	2.42	0.36148	1.15	2.12	1.6440	0.35874
PVD	27.80	42.70	33.88	5.38017	16.29	22.38	19.5940	1.89574
BD	10.30	13.80	11.20	1.02524	4.11	6.17	5.1240	0.68159
CPD	6.60	10.20	8.39	1.29224	5.12	7.57	6.0320	0.77787

Molecular study. A small piece of pelvic fins of 13 specimens of *Paraschistura* were preserved in alcohol for molecular survey. A small piece of dorsal muscle of each alcohol

preserved fish was removed in sterile condition and placed in 96-well Eppendorf PCR plates until further processing. Total genomic DNA was purified by extraction of tissues using the salt method (Bruford et al. 1992). The entire COI gene was amplified via PCR using the primers (VF1d 5ʹTTCTCAACCAACCACAARGAYATYG G3ʹ, VR1d 5ʹ TAGACTTCTGGGTGGC CRAA- RAAYCA3ʹ) (Ivanova et al. 2006). Amplification of COI was performed under the following temperature regime: initially 94ºC for 3 min, 35 cycles at 94ºC for 30 sec, 58ºC for 30 sec and 72ºC for 90 sec, and a final extension step at 72ºC for 7 min. The COI gene was sequenced using dye-labeled dideoxy terminator cycle sequencing with Big Dye v.3.1 (Applied Biosystems, Inc). Sequences were aligned with the Clustal W algorithm (Thompson et al. 1997), using BioEdit v.7.0.5 (Hall 1999) and adjusted by CLC Main Workbench 5.5 (CLC bio, Katrinebjerg 8200, Aarhus N, Denmark). The resulting COI sequences are deposited in GenBank under accession numbersKY305500-KY305512.

An additional 119 sequences from ten species of *Paraschistura* in Genbank were used in order to obtain a representative dataset for assessment of the phylogenetic position of the new specimens and to compare phylogenetic tree by that of Freyhof et al. (2015) (Table 4). *Turcinoemacheilus hafezi* was selected as an outgroup based on studies of Freyhof et al. (2015) and Jouladeh-Roudbar et al. (2015). Three sequences that Jouladeh-Roudbar et al. (2015) present in their study are not available in GenBank so we could not use them in our phylogenetic tree.

Maximum likelihood reconstructions were performed using RAxML 7.2.5 (Stamatakis 2006). Support for internal branches was evaluated by non-parametric bootstrapping with 2000 replicates. For this dataset, best fit combinations of partitioning schemes and nucleotide substitution models were determined with Partition Finder version 1.0.1 (Lanfear et al. 2012), using the ''greedy'' algorithm and the Bayesian information criterion (BIC), with the branch lengths of alternative partitions linked and with the software set to evaluate specific substitution model sets for either RAxML (GTRGAMMAI) or MrBayes (K80GAMMAI) independently. Phylogenetic relationships based on Bayesian inference (BI) was estimated using MrBayes 3.2 (Ronquist et al. 2012) with two runs of four Markov Chain Monte Carlo (MCMC) chains over a total 10,000,000 generations. Chains were stopped after the standard deviation of split frequencies fell below 0.01, and the likelihood values of sampled trees from both runs reached a stationary distribution. The first 25% of trees before were disregarded as "burnin".

Results
Obtained results showed that the genus *Paraschistura* in Hari River contains at least two species, *Paraschistura cristata* and *Paraschistura turcmenica.* Ten specimens from every species were measured and counted for morphometric and meristic characters (Tables 2, 3).

Paraschistura cristata (Berg, 1898)
(Fig. 1)
Nemachilus cristatus Berg, 1898
Material examined. 75 specimens, 52.34-71.02 mm SL; Iran: Khorasan Razavi Prov.: Pole-Khatun, Darungar, Radkan, Abghad, Kleilagh, Sarasiab (Table 1); coll. M. Ghanbarifardi, R.A. Khazaee. June, August and October 2013, (Fish section) ZMFUM.PCRa 1-10.
Diagnosis. A prominent dorsal adipose crest supported by 22-25 procurrent rays of the caudal fin and a complete lateral line. 8½ branched dorsal-fin rays; spot at base of first dorsal-fin rays bold; pelvic fin reaching to or slightly in front of anus (see Freyhof et al. 2015).
Distribution. *Paraschistura cristata* is known from the Hari River basin in Afghanistan, Iran and Turkmenistan.

Paraschistura turcmenica (Berg, 1932)
(Fig. 2)
Material examined. 90 specimens, 30.29-40.53 mm SL; Iran: Khorasan Razavi Prov.:

Pole-Khatun, Radkan, Baghe-Khan, Abghad, Kleilagh, Sarasiab (Table 1); coll. M. Ghanbarifardi, R.A. Khazaee. June and August 2013, (Fish section) ZMFUM.PTKl 1-10.

Table 3. Species of *Paraschistura* used in this study from GenBank.

Species	No.	Accession numbers
Paraschistura abdolii	22	KM603314, KM603307, KM603304, KM603303, KM603300,
Paraschistura cristata		KM603312, KM603293, KM603282, KM603281, KM603262, KM603229, KM603223, KM603203
Paraschistura naumanni	15	KM603311, KM603309, KM603297, KM603288, KM603285, KM603272, KM603269, KM603268, KM603265, KM603252, KM603251, KM603226, KM603220, KM603215, KM603200
Paraschistura turcmenica	7	KM603305, KM603302, KM603286, KM603248, KM603245,
Paraschistura aredvii	7	KM603301, KM603289, KM603274, KM603270, KM603256, KM603236, KM603212
Paraschistura hormuzensis	2	KM603296, KM603202
Paraschistura bampurensis	19	KM603294, KM603291, KM603284, KM603277, KM603276, KM603266, KM603264, KM603263, KM603247, KM603241, KM603240, KM603231, KM603230, KM603222, KM603218, KM603214, KM603210, KJ179269, KJ179268
Paraschistura susiani	9	KM603290, KM603283, KM603258, KM603250, KM603237, KM603232, KM603225, KM603221, KM603209
Paraschistura nielseni	8	KM603306, KM603287, KM603280, KM603275, KM603257, KM603234, KM603217, KM603211
Paraschistura pasatigris	17	KM603313, KM603310, KM603308, KM603295, KM603279, KM603273, KM603267, KM603261, KM603260, KM603259, KM603255, KM603239, KM603238, KM603228, KM603224, KM603204, KM603201
Paraschistura sp.	3	KM603278, KM603254, KM603235
Turcinoemacheilus hafezi	2	KJ179264, KJ179252

Fig. 1. *Paraschistura turcmenica* (Sarhadd Loach) from Abghad. SL: 40.5 mm.

Fig. 2. *Paraschistura cristata* (Turkmenian Crested Loach) from Abghad. SL: 59.2 mm.
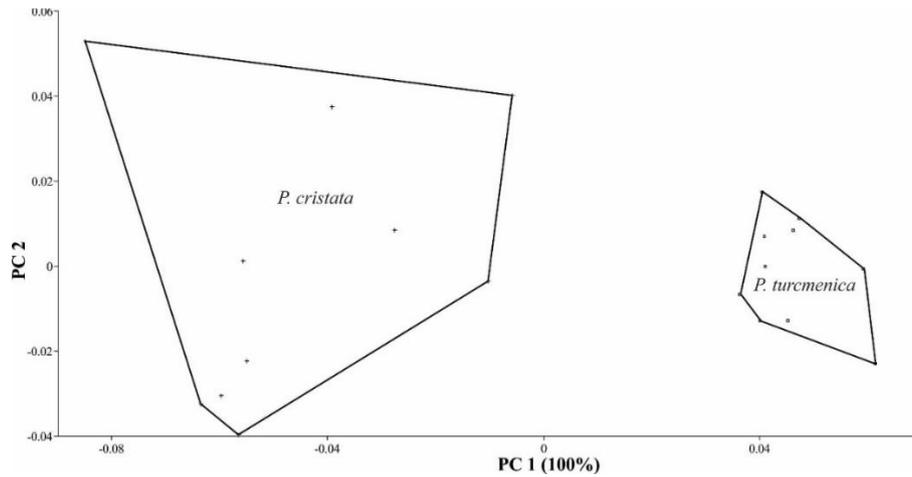
Fig. 3. Scatterplot of Principal Component Analysis (PCA) based on eight meristic and nineteen morphometric characters for two examined species (*P. cristata* and *P. turcmenica*).
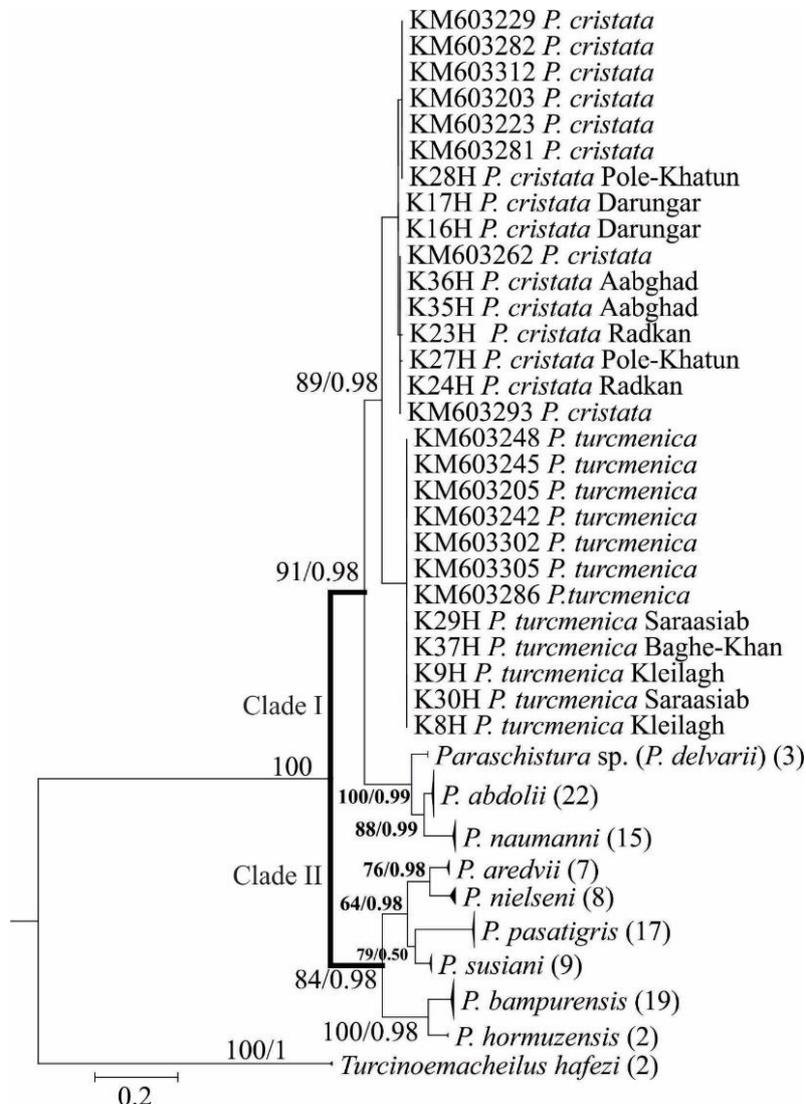

Fig. 4. Maximum likelihood estimate (based on COI gene) of phylogenetic relationships of the different species of *Paraschistura*. Numbers above branches represent maximum likelihood bootstrap values based on 2000 replicates followed by Bayesian posterior probabilities. Number in the parenthesis represent the number of sequences used for every species.

Diagnosis. Body is deep with a color pattern consisting of vague dark brown spots on flank, tending posteriorly to form transverse bars in both small and large specimens in life and preserved, deeper body and caudal peduncle, 7½ branched rays in dorsal fin, and incomplete lateral line (see Freyhof et al. 2015).

Distribution. Found in the western Hari River basin in Turkmenistan and Iran. Eight meristic characters and nineteen morphometric characters could recognize *P. cristata* and *P. turcmenica* clearly based on PCA (Fig. 3). The first Principal component (PC) accounted for 100% of total variance. For molecular study 13 specimens of *Paraschistura* were sequenced from Khorasan-Razavi, Iran. These fish were collected from seven stations namely Pole-Khatun, Darungar, Radkan, Abghad, Baghe-Khan, Kleilagh and Sarasiab. All of our specimens have been classified with *P. cristata* and *P. turcmenica* which are presented in Freyhof et al. (2015) (Fig. 4). The maximum likelihood and Bayesian trees are congruent and clearly indicate that there are two main clades in phylogenetic tree; Clade I, *P. cristata, P. turcmenica, P.* sp. (*P. delvarii*), *P. naumanni* and *P. abdolii*; Clade II, *P. hormuzensis, P. bampurensis, P. nielseni, P. aredvii, P. pasatigris* and *P. susiani*. Moreover, the tree reveals that *P. delvarii* is a sister taxon for the clade which is composed of *P. naumanni* and *P. abdolii*.

Discussion and Conclusions

According to Mousavi Sabet et al. (2015), Freyhof et al. (2015) and our examined materials, *Paraschistura turcmenica* is distinguished from the other species of by a color pattern in both live and preserved specimens consisting of vague dark brown spots on flank, tending posteriorly to form transverse bars, 7½ branched rays in dorsal fin, elongated and shallow body and caudal peduncle and *Paraschistura cristata* is distinguished from the congeners in the Hari River basin by having a prominent dorsal adipose, complete lateral line 9-13 brown bars usually faded or absent on flank in front of dorsal fin origin,; no suborbital groove or flap in males; usually 8½ branched dorsal-fin rays; spot at base of first dorsal-fin rays bold; pelvic fin reaching to or slightly in front of anus.

Traditional methods of identifying, naming and classifying fishes are mainly employed visible morphology. However, modern taxonomic work recognizes Cytochrome Oxidase Subunit I as a molecular tool for barcoding of fishes and phylogenetic approaches (Ward et al. 2009). In this study COI has been used for molecular identification of *P. cristata* and *P. turcmenica*. Moreover, getting access to COI sequences, we could compare our results with other major studies on *Paraschistura* which have used COI for DNA barcoding and phylogenetic survey (Freyhof et al. 2015; Jouladeh-Roudbar et al. 2015). The phylogenetic tree (Fig. 4) presented in this study is largely congruent with that of Freyhof et al. (2015). Freyhof et al. (2015) identify a clade composed of *P. pasatigris* and *P. susiani* which its sister taxa bear *P. hormuzensis* and *P. bampurensis*. However, our phylogenetic tree identifies *P. aredvii* and *P. nielseni* as sister taxa of *P. pasatigris* and *P. susiani*. *Parascistura nielseni* (Persis sub-basin), *P. aredvii* (Zohreh sub-basin), *P. pasatigris* (Tigris basin) and *P. susiani* (Tigris basin) are recorded and sequenced from neighboring sub-basins that are belong to long Persian Gulf basin (Esmaeili et al. 2016) and more importantly, our phylogenetic tree put these four species in one clade. Moreover, Jouladeh-Roudbar et al. (2015) agree with us to put these four species from Persian Gulf basin (*P. nielseni*, *P. aredvii*, *P. pasatigris* and *P. susiani*) in one clade. *Paraschistura* sp. (*Paraschistura delvarii*) is the sister taxon of *P. naumanni* and *P. abdolii* (Mousavi-Sabet & Eagderi 2015). The phylogenetic tree of this study and phylogenetic analyses of recent studies (Freyhof et al. 2015; Jouladeh-Roudbar et al. 2015) are based on COI. To get a deeper view in evolution and speciation of this genus, *Paraschistura*, sequencing at least one nuclear gene is needed in future studies.

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مقاله پژوهشی

سیستماتیک مولکولی جنس *Paraschistura* (Teleostei: Nemacheilidae) در حوضه آبریز هریرود

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چکیده: سگ ماهیان جنس *Paraschistura* به طور گسترده ای از حوضه آبریز تیگره در ترکیه و ایران تا حوضه های آبریز رودخانه‌های سند، هریرود، مرغاب و هلمند در افغانستان، ایران، پاکستان و ترکمنستان پراکنش یافته‌اند. نمونه‌های *Paraschistura* از هفت ایستگاه در حوضه‌های آبریز هریرود با استفاده از تور دستی جمع‌آوری و توالی‌های COI برای دو گونه *Paraschistura cristata* و *Paraschistura turcmenica* ارائه شده‌اند. به علاوه، ۱۱۹ توالی مربوط به ده گونه از *Paraschistura* موجود در بانک ژن به توالی‌های جدید اضافه گردید تا موقعیت تبارزایی نمونه‌های *Paraschistura* مشخص و درخت تبارزایی حاصل با مطالعات دیگر مقایسه شود. نتایج مولکولی حاصل از درخت حداکثر احتمال (ML) و درخت بیزین (BI) متجانس بوده و به طور واضحی دو دودمان اصلی را نشان می‌دهند. نتایج حاصله حضور دو گونه *P. cristata* و *P. turcmenica* را در حوضه آبریز هریرود تأیید می‌نماید.

کلیدواژه‌ها: *Paraschistura cristata, Paraschistura turcmenica*، توالی COI، خراسان رضوی، ایران