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Molecular validity of nemacheilid loaches from Choman River drainage, Kurdistan, Iran

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Abstract

The validity of three morphologically described loach species viz. *Oxynoemacheilus chomanicus*, *O. zagrosensis*, *O. kurdistanicus* and one geographically newly reported species, *Turcinoemacheilus kosswigi* from Choman River drainage, in western Iran were evaluated using Cytochrome *b* gene. The results confirmed the validity of four Choman River's loaches as distinct species and the four species were clustered well within *Oxynoemacheilus* and *Turcinoemacheilus* genera. Based on the results, Cytochrome *b* gene was useful tool for identification of Choman River's loaches.

Keywords: Sequences, Classification, Phylogenetic relationship, Taxonomy.

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Introduction

Loaches of the family Nemacheilidae are a diverse lineage inhabiting lotic freshwaters of Asia, Europe, and north-eastern Africa with more than 750 species so far (Eschmeyer et al. 2020). The family is well-represented in Iran with more than 46 species (Abdoli et al. 2011; Golzarianpour et al. 2013; Kamangar et al. 2014; Freyhof et al. 2016; Sayyadzadeh et al. 2016; Esmaeili et al. 2018). Although, their validity and taxonomic status are disputed since many species of this family described based on solely morphological features (Azimi et al. 2015; Sungur et al. 2017; Cicek et al. 2018) but recent advancement in molecular studies, helped to resolve their correct taxonomic status (Chaudhary and Singh 2012; Huang et al. 2016, 2017). Molecular methods have great potential for resolving identification and phylogenic relationships of fish species e.g. in cryptic species (Teletchea 2009). Among many genes utilized in phylogenetic studies, Cytochrome *b* (Cytb) is a frequently one in fish identification (Baharum and Nurdalila 2011; Biswas et al. 2001; Perdices et al. 2004; Perkins and Schall 2002). Within family Nemacheilidae, Cytb has been well-used to identify new species and to some extent establish phylogenetic relationship (Lokeshwor et al. 2012; Min et al. 2012). However, this gene also has limitations which includes base compositional biases, rate variation between different lineages, early saturation of third codon positions, and limited variation in the first and second codon positions (Meyer 1993). Therefore, questions regarding the deep phylogenetic relationship at the higher classification cannot be accurately answered using this gene.

Recently, three stone loaches of Nemacheilidae family have been described from Choman River drainage viz. *Oxynoemacheilus chomanicus* Kamangar et al. 2014, *O. zagrosensis* Kamangar et al. 2014, and *O. kurdistanicus* Kamangar et al. 2014 based on their morphological characters (Kamangar et al. 2014). Additionally, a new geographical record has been reported for *Turcinoemacheilus kosswigi* Bănărescu and Nalbant, 1964 from the same drainage. These new findings presented an opportunity for us to assess their validity using Cytochrome *b* gene as aim of this study.

Material and Methods

Twenty specimens (5 specimens for each species) of *O. chomanicus* (38.1-56.2 mm SL) from three localities,

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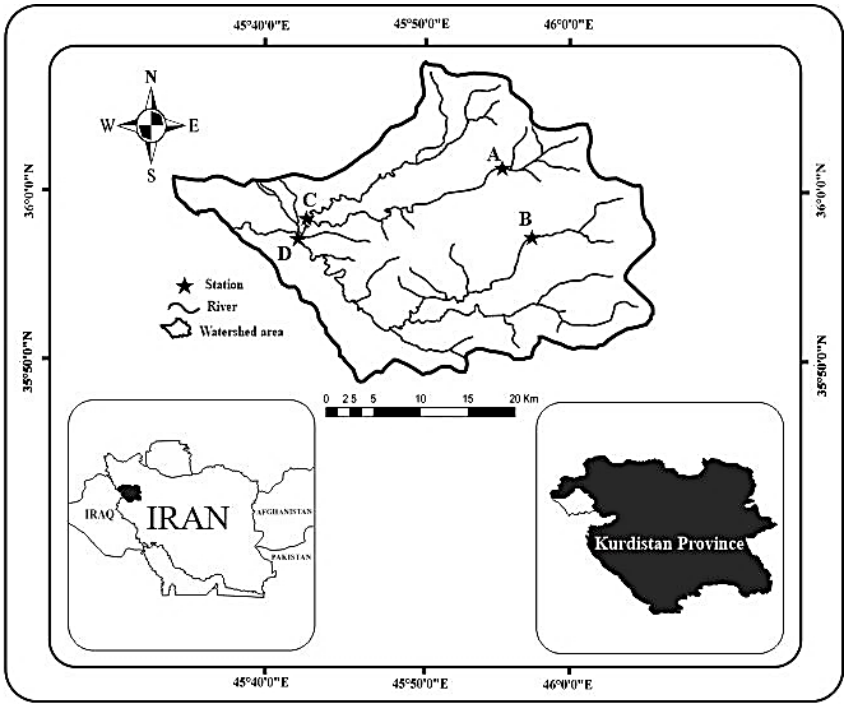

Figure 1. Map showing the location of samplings (A) Korhe-pazi at Baneh River (36º01′03N, 45º55′20″E), (B) Payepol at Boein River (35º56′30″N, 45º56′36″E), (C) Jemli at Choman River (35º57′54″N, 45º42′30″E) and (D) Tajaban at Choman River (35º56′53″N, 45º41′40″E).

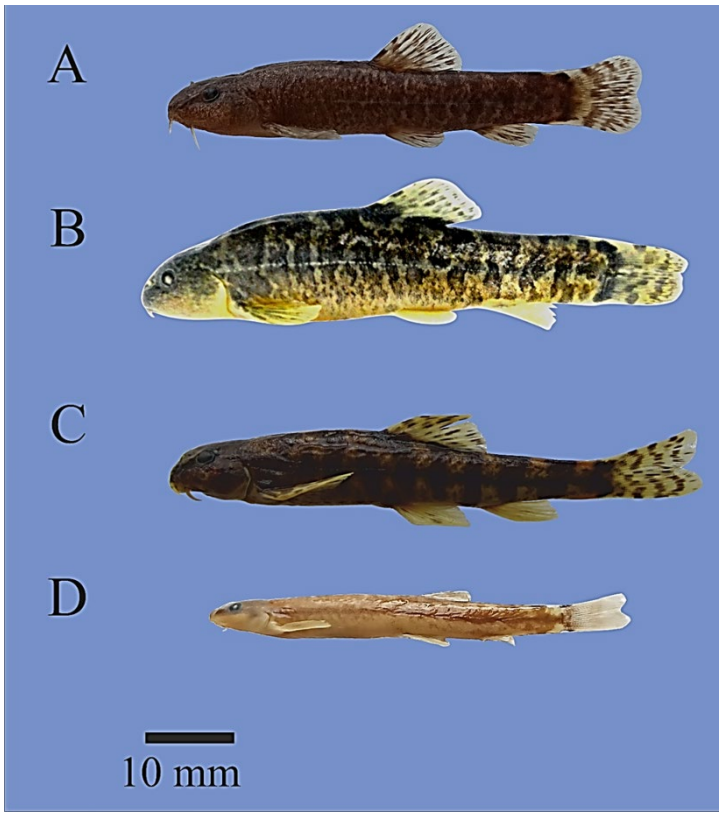

Figure 2. (A) *Oxynoemacheilus chomanicus*, (B) *O. zagrosensis*, (C) *O. kurdistanicus* and (D) *Turcinoemacheilus kosswigi*.

including (station A in Fig. 1) Korepazi in Baneh River (n=2), (B) Payepol in Boein River (n=1) and (C) Jemli in Choman River (n=2), *O. zagrosensis* (48.4-58.4 mm SL) from two localities in (C) Jemli in Choman River (n=2) and (D) Tajaban in Choman River (n=3), *O. kurdistanicus* (32.2-60.5 mm SL) and *T. kosswigi* (25-49.4 mm SL) from Tajaban in Choman River (D) were collected in August and September 2011 (Fig. 2). All specimens fixed in ethanol 96% after anesthetized with clove oil and samples of their muscles tissue were taken from for molecular study.

Table 1. Cytochrome *b* sequences downloaded from NCBI GenBank with information on drainage, country of origin and source.

Species	Drainage	Country	Published by	Genbank Acc. No.
Oxynoemacheilus zarzianus	Lesser Zab	Iraq	Freyhof and Geiger 2017	KY849792
Oxynoemacheilus zarzianus	Lesser Zab	Iraq	Freyhof and Geiger 2017	KY849795
Oxynoemacheilus zarzianus	Lesser Zab	Iraq	Freyhof and Geiger 2017	KY849796
Oxynoemacheilus zarzianus	Lesser Zab	Iraq	Freyhof and Geiger 2017	KY884997
Oxynoemacheilus bureschi	Balkan		Sediva et al. 2010	GQ199473
Oxynoemacheilus bureschi	Balkan		Sediva et al. 2010	GQ199474
Oxynoemacheilus bureschi	Balkan		Sediva et al. 2010	GQ199475
Oxynoemacheilus bureschi	Balkan		Sediva et al. 2010	GQ199476
Turcinoemacheilus sp.	Karoun	Iran	Jamshidi et al. 2013	GQ338826
Turcinoemacheilus sp.	Karoun	Iran	Jamshidi et al. 2013	GQ338827
Turcinoemacheilus sp.	Karoun	Iran	Jamshidi et al. 2013	GQ338828
Oxynoemacheilus hanae	Sirvan	Iraq	Freyhof and Abdullah 2017	MH842969
Oxynoemacheilus gyndes	Sirvan	Iraq	Freyhof and Abdullah 2017	MH842968
Paracobitis atrakensis	Kavir basin	Iran	Sayyadzadeh et al. 2019	MG229862
Paracobitis atrakensis	Atrak	Iran	Sayyadzadeh et al. 2019	MG229861
Paracobitis atrakensis	Atrak	Iran	Sayyadzadeh et al. 2019	MG229863
Paracobitis hircanica	Zarin-Gol	Iran	Sayyadzadeh et al. 2019	MG229864
Paracobitis hircanica	Zarin-Gol	Iran	Sayyadzadeh et al. 2019	MG229865
Paracobitis malapterura	Namak	Iran	Sayyadzadeh et al. 2019	MG229878
Paracobitis malapterura	Qom	Iran	Sayyadzadeh et al. 2019	MG229879
Paracobitis malapterura	kordan	Iran	Sayyadzadeh et al. 2019	MG229880
Paracobitis malapterura	Kavir basin	Iran	Sayyadzadeh et al. 2019	MG229881
Paracobitis molavii	Lesser Zab	Iran	Sayyadzadeh et al. 2019	MG229860
Paracobitis persa	Kor	Iran	Sayyadzadeh et al. 2019	MG229866
Paracobitis persa	Kor	Iran	Sayyadzadeh et al. 2019	MG229868
Paracobitis rhadinaea	Sistan	Iran	Sayyadzadeh et al. 2019	MG229876
Paracobitis rhadinaea	Sistan	Iran	Sayyadzadeh et al. 2019	MG229877
Triplophysa leptosoma	Qilian	China	Zhang et al. 2017	KX213666

DNA was extracted using salting out protocol from muscle tissue (Cawthorn et al. 2011). To amplify a 1140 bp of Cytochrome *b*, DNA extracts were subjected to PCR amplification, using primer set of L14724 (5'-GA CTTGAAAAACCACCGTTG-3') and H15915 (5'-CTCCGATCTCCGGATTACAAGAC-3') (Tang et al. 2006). PCR was performed at an initial denaturation step at 94˚C for 3 min followed by 35 cycles at 94˚C for 30 s, 52-58˚C for 45 s, 72˚C for 1 min and a final extension at 72˚C for 8 min. The amplified fragments were purified with Promega DNA purification kit following the manufacturer's instructions. The purified fragments were sequenced bidirectional by BIONEER Company. All sequences were deposited the GenBank database.

Kimura two parameter (K2P) distance model (Kimura 1980) was used to calculate sequence divergences in Choman's loach species. Two categories of K2P were calculated to test intra and interspecific distances in Choman's loach species. K2P genetic distances were calculated using MEGA 6 software package (Tamura et al. 2013). For phylogenetic analysis, 20 sequences from Choman's loach species were included with 27 sequences representing 11 species of Nemacheilidae family (Table 1). *Triplophysa leptosoma* (KX213666) was used as out-group. Our attempt to obtain the full fragment of cytochrome *b* genes was not successful and the samples obtained were read incompletely with the H15915 primer. The average sequence length was 1097 bp within the range of 1011 to 1131 bp. For this reason, we considered the sequences of Choman and extracted the sequences from the gene bank with 989 bp nucleotide fragments.

Phylogenetic trees were reconstructed for the gene data set using Neighbor Joining (NJ), Maximum Likelihood (ML) and Bayesian Analyses (BA). Sequences were aligned using Clustal X ver. 1.85 (Thompson et al. 1997). NJ phylogeny was inferred with neighbor-joining algorithm and bootstrapping support for the nodes

Table 2. Mean interspecific K2P genetic distances between the Choman loach species.

	O. chomanicus	*O. zagrosensis*	*O. kurdistanicus*
O. chomanicus			
O. zagrosensis	4.8%		
O. kurdistanicus	3.7%	6.6%	
T. kosswigi	22.7%	24.1%	23.9%

(10,000 pseudoreplicates) as implemented in PAUP* 4.0b10 (Swofford 2002; Windows version). Phylogenetic trees using ML were estimated as implemented in RAxML (Randomized Axelerated Maximum Likelihood, version 7.0.4) (Stamatakis 2006). The Akaike information criterion (AIC) implemented in MODELTEST v3.4 (Posada and Crandall 2003) was used to identify the optimal molecular evolutionary model for each partition on the sequence data set. GTR+I+G model were utilized to obtain the optimal ML trees and bootstrap support. Robustness of the inferred trees was evaluated using bootstrap analysis on 10,000 pseudoreplications using RAxML 7.0.4 (Felsenstein 1985; Stamatakis et al. 2008). BA analyses were conducted using Mr. Bayes v3.1.2 (Huelsenbeck and Ronquist 2001). For BA, 10,000,000 cycles were implemented for four simultaneous Monte Carlo Markov chains, sampling the Markov chain at intervals of 100 generations. Log-likelihood stability was attained after 100,000 generations; the first 1,000 trees were discarded as "burn-in" in each analysis. Support for BI tree nodes was determined based on values of Bayesian posterior probabilities. Final trees were visualized in the FigTree v.1.4.2 program (Rambaut 2019).

Results

Of the total 989 sites, 373 sites were variable, and 311 sites were parsimony informative. No internal indels were observed in any sequences generated herein or downloaded from the Genebank. The average nucleotide composition of *O. chomanicus*, *O. zagrosensis*, *O. kurdistanicus* and *T. kosswigi* sequences were Adenine (A) 25.2, 24.9, 24.9 and 27.9%, Thymine (T) 31.4, 31, 30.7 and 31.5%, Cytosine (C) 26.8, 27, 27.5 and 24.8% and Guanine (G) 16.6, 17.1, 16.9 and 15.7%, respectively. In all four species, the content of A+T was higher than that of C+G. Transitions (Ti) / transversions (Tv) ratios for *O. chomanicus*, *O. zagrosensis*, *O. kurdistanicus* and *T. kosswigi* were 2.21, 1.27, 0.965 and 2.1, respectively. Intraspecific K2P distance for Choman's loach species were 0.7, 0.9, 0.3 and 1% for *O. chomanicus*, *O. zagrosensis*, *O. kurdistanicus* and *T. kosswigi*, respectively. The highest interspecific distance (24.1%) was observed between *T. kosswigi* and *O. zagrosensis*, whereas the lowest distance (3.7%) was observed between *O. chomanicus* and *O. kurdistanicus* (Table 2).

The obtained trees from three analyses, NJ, ML and BI demonstrated an identical relationship (Fig. 3). The phylogenetic trees divided all specimens into two clades. Clade one contained *Oxynoemacheilus* sequences including those from Choman and the other clade consisted of loaches of *Turcinoemacheilus* sequences including *T. kosswigi*. Among *Oxynoemacheilus* species, the Choman loaches of *O. zagronensis* and *O. chomanicus* seems to be more closely related.

Discussion

Identification of loach species has always been a difficult task and their morphological classification has shown instability (Regan 1911; Hora 1932; Sawada 1982; Siebert 1987). The problems in this regard appear in two forms, differentiating specimens from the same species and to a lesser extent similarity between specimens of different species. Therefore, in this study, we employed a more reliable method to confirm the loach species in the work of Kamangar et al. (2014). Based on the results, the Cyb sequences of *T. kosswigi* from the Choman

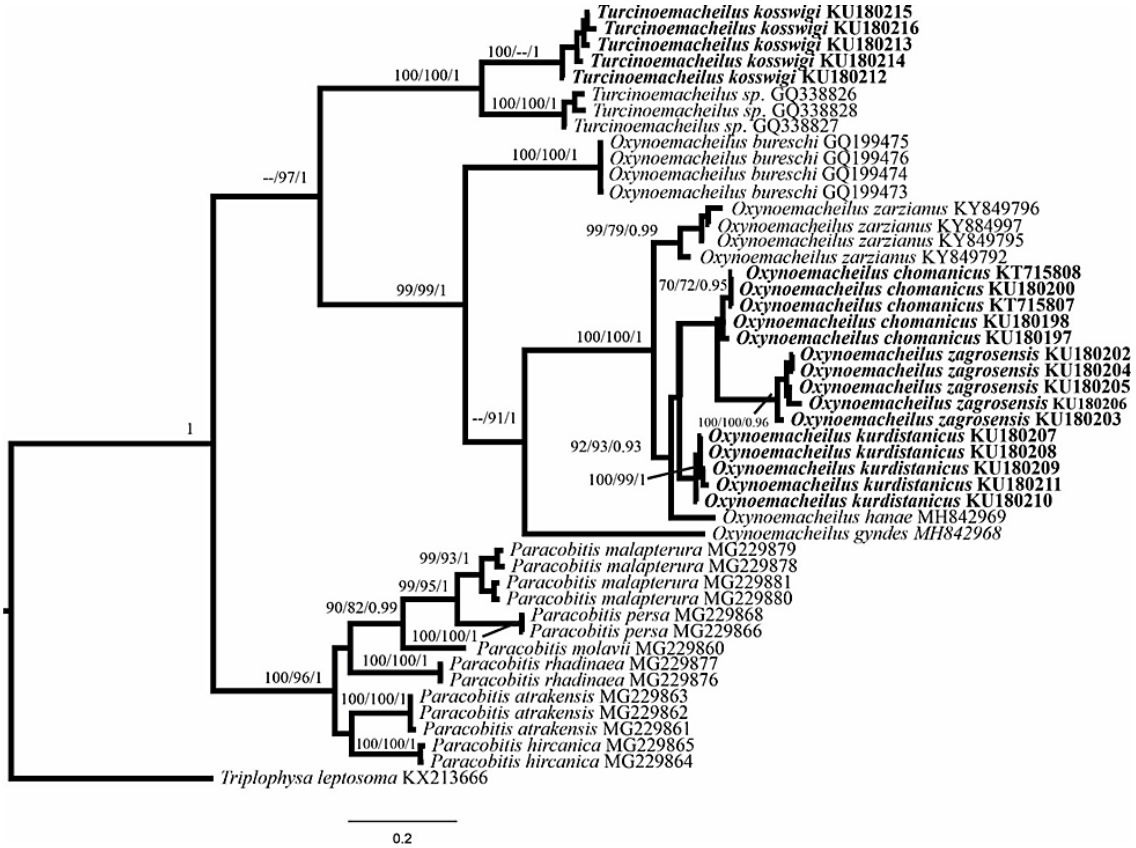

Figure 2. Phylogenetic tree of the studied Choman loaches species. Similar tree topologies were supported by NJ, ML and Bayesian inference methods. Numbers on nodes represent NJ and ML bootstrap support for that node (number of pseudoreplicates 10000 for NJ and ML) and posterior probabilities for BI (number of generations 10,000,000), respectively. The names of Choman loaches species are depicted in bold.

river drainage shows similarity to *Turcinoemacheilus* sp. sequences as it was reported from Iran by Jamshidi et al. (2013) (Accessions; GQ 338826, GQ 338827 and GQ 338828). Jamshidi et al. (2013) suspected that these sequences belong to the *Turcinoemacheilus kosswigi*. Our findings did not confirm their results. The results revealed that the *T. kosswigi* and *Turcinoemacheilus* sp. are probably two distinct taxa.

The k2p genetic distance between Choman loaches of the genus *Oxynoemacheilus* were less than 1% and for *T. kosswigi* at 1% too. The results were in agreement with by Li et al. (2018) that Cytb intraspecific differences in fishes (i.e. amount of K2P) are generally less than 1%, while interspecific differences are generally higher than 10%. Based on the results, within the *Oxynoemacheilus* clade, two clades are detected including (1) those of our materials as well as *O. hanae* and *O. zarzianus* and (2) *O. gyndes*. A recent study by Feryhof and Abdullah (2017) has shown that *O. kurdistanicus* is sympatric with those of *O. hanae* and *O. gyndes.* Our results have shown that Cytb genes are useful in identification of the Choman loaches and confirmed their validity. Further, the validity and distinct clade of the loach *T. kosswigi* is supported.

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