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A DNA barcoding approach to identify species of Nemacheilidae: a complex family of cypriniform fishes in Iran

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Nemacheilian loaches are a group of freshwater fishes which are distributed in many inland waters of Iran. They are complex species from aspects of systematic with many species of this family being morphologically undistinguishable. Due to limitations behind the morphology-based identification of such species, genetic approaches such as DNA barcoding can be helpful to distinguish species, although their genetic relationships are not well determined yet. The present investigation provides data on genetic structure of some species of Nemachilidae including *Paraschistura bampurensis*, *Oxynoemacheilus kiabii* and *Turcinemacheilus saadii* from inland waters of Iran. These species were sequenced for a 652 base pair region of the mitochondrial cytochrome oxidase subunit I gene. All species were identified by cytochrome oxidase subunit I gene sequence. The sequences of *P. bampurensis* showed that specimens collected from two streams (Shapour and Fahlian, south-west of Iran), with 1.89% within-species Kimura two parameter distance, shared haplotype in neighbor joining tree. The results provided the first genetic evidence for *O. kiabii,* which has recently been recorded as a new species in Iran, and also cytochrome oxidase subunit I gene data showed that *O. kiabii* is completely different from other species of Nemacheilidae. The results suggested that cytochrome oxidase subunit I gene sequencing can be used to identify species of Nemachilidae from the Iranian streams. The picture of Nemachilidae is yet incomplete, and this study is one of the first steps towards enhancing genetic understanding of associations among the species of Nemachilidae in Iran. There should also be further attempts in genetic or systematic research to fill data gaps among fish species in Iran to help establishing a global COI barcode database.

Key words: *Cytochrome oxidase, DNA barcoding, Nemachilidae, phylogenetic tree, species identification.*

INTRODUCTION

Nemacheilian loaches are benthic species that are distributed in many inland waters of Europe, northeast Africa and Asia including streams of Iran (Abdoli, 2000; Coad, 2016), however, their characteristics are not well described. Although species of Nemacheilidae are not commercially important, the family is of high ecological significance and is a main part of river ecosystems in the region. In western Asia, the nemacheilid loaches include numerous species that are morphologically

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similar. Recent findings regarding Nemacheilidae have led to the identification of new species in Iran (Coad & Nalbant, 2005; Golzarianpour *et al.*, 2011a, 2011b, 2013; Esmaeili *et al.*, 2014), which means there is still considerable ambiguity in terms of identifying species of Nemachilidae. In other words, what is known about them, is mostly based on limited data, and previous studies have mainly focused on morphometric aspects of the species from this family (Askari & Shabani, 2013; Esmaeili *et al.*, 2013; Kolangi-Miandare *et al.*, 2013).

Nalbant and Bianco (1998) reported at least 3 genera and 14 species of nemacheilid loaches from Iran. However, recent investigations revealed that the species from this family are more diverse than the numbers previously known, with evidence reporting over 40 species including 24 loach species endemic to Iran (Abdoli *et al.*, 2011; Esmaeili *et al.*, 2014, Freyhof *et al.*, 2014; Mousavi Sabet *et al.*, 2015). Despite recent efforts in identifying these fishes, the list is predicted to be incomplete and many taxonomic difficulties remain at the species level mainly due to their small size and low commercial value (Tang *et al.*, 2006; Kottelat, 1990). Nonetheless, delineation and identification of fish species is a requirement in studies of ecology, natural history and fisheries management. Considering the limitations of anatomical and morphological methods of species identifications such as the occurrence of a large morphological plasticity or similarity between organisms of the same species, the availability of a small amount of biological material and the existence of convergent evolution (Pereira *et al.*, 2008; Cawthorn *et al.*, 2011), using more accurate and cost effective approaches is suggested.

With the development of a new genetic techniques, the field of species identification have relayed on the information from the molecular components of cell. Molecular methods and their application for genetic conservation and species differentiation studies have been promoted remarkably over the past 20 years (Zhang *et al.*, 2004; Comi *et al.*, 2005; Teletchea, 2009; Dettai *et al.*, 2011, Chiu *et al.*, 2012). DNA-based approaches are among the most used methods for molecular species identification (Shendure *et al.*, 2004; Kochzius *et al.*, 2010; Zhang & Hanner, 2012). The method has been suggested to facilitate the process of species discovery as well as to assign unknown individuals to species (Hajibabaei *et al.*, 2007; Herbert & Gregory, 2005, Mohanty *et al.*, 2015).

The term DNA barcoding is generally considered as using a short section of DNA from a standardized region of the genome for species identification (Hebert *et al.*, 2003a; Hebert *et al.*, 2003b; Hebert & Gregory, 2005). The aim of DNA barcoding is producing a database for origins of species and also related sequence information of a 648 base pair (bp) segment of mitochondrial cytochrome c oxidase I (CO1), and classifying an organism on the basis of a short sequence of its mitochondrial DNA (mtDNA) (Hebert *et al.*, 2003a, 2003b; Moritz & Cicero, 2004; Wong *et al.*, 2011). The mtDNA has many uses in the field of evolution because of its higher mutation rate and lower effective population size compared to nuclear DNA (Brown *et al.*, 1979). The COI is proposed as a standard barcode for animals (Hebert *et al.*, 2003a) and could identify a large variety of species (Steinke *et al.* 2005, 2009; Pegg *et al.* 2006). DNA barcoding is also a helpful approach to find new species of threatened freshwater fishes (Torres *et al.*, 2013). Moreover, mtDNA sequences can be utilized for studying population genetics and phylogenetics of fish (Peng *et al.*, 2004; Liu & Chen, 2003).

Therefore, considering difficulties in the identification of Nemacheilidae species based on their morphological characteristics, the aim of this study was to provide barcoding data for some species of Nemachilidae in order to rapid and accurate species identification as well as looking forward to find internal species marker. Hence, this paper attempts to study the genetic situation of some Nemacheilidae species, and to prepare DNA barcoding to compare the relationship among species.

MATERIAL AND METHODS

Study area and fish collections

Nemacheilian loaches were sampled from Shapour, Fahlian and Gamasyab Rivers in the west and southwest of Iran (Fig. 1). Shapour and Fahlian Rivers are two important permanent rivers in Fars province as these rivers are the main water sources in the region, especially for agricultural activities. Gamasyab River is one of the longest permanent rivers in Iran. It originates from the northern slopes of Garin Mountain called the Garin headwater. It flows through a few provinces (Hamedan, Kermanshah, Lorestan and khuzestan) and ends to the Hoor Al-Azim international wetland. Three Nemacheilian loaches included *Paraschistura bampurensis* from Shapour and Fahlian rivers, and *Oxynoemacheilus kiabii* and *Turcinemacheilus saadii* from Gamasyab River (Fig. 2). The fish were collected using nets during May to July, 2012. All specimens were fixed in ethanol (96%) and then transferred to the lab.

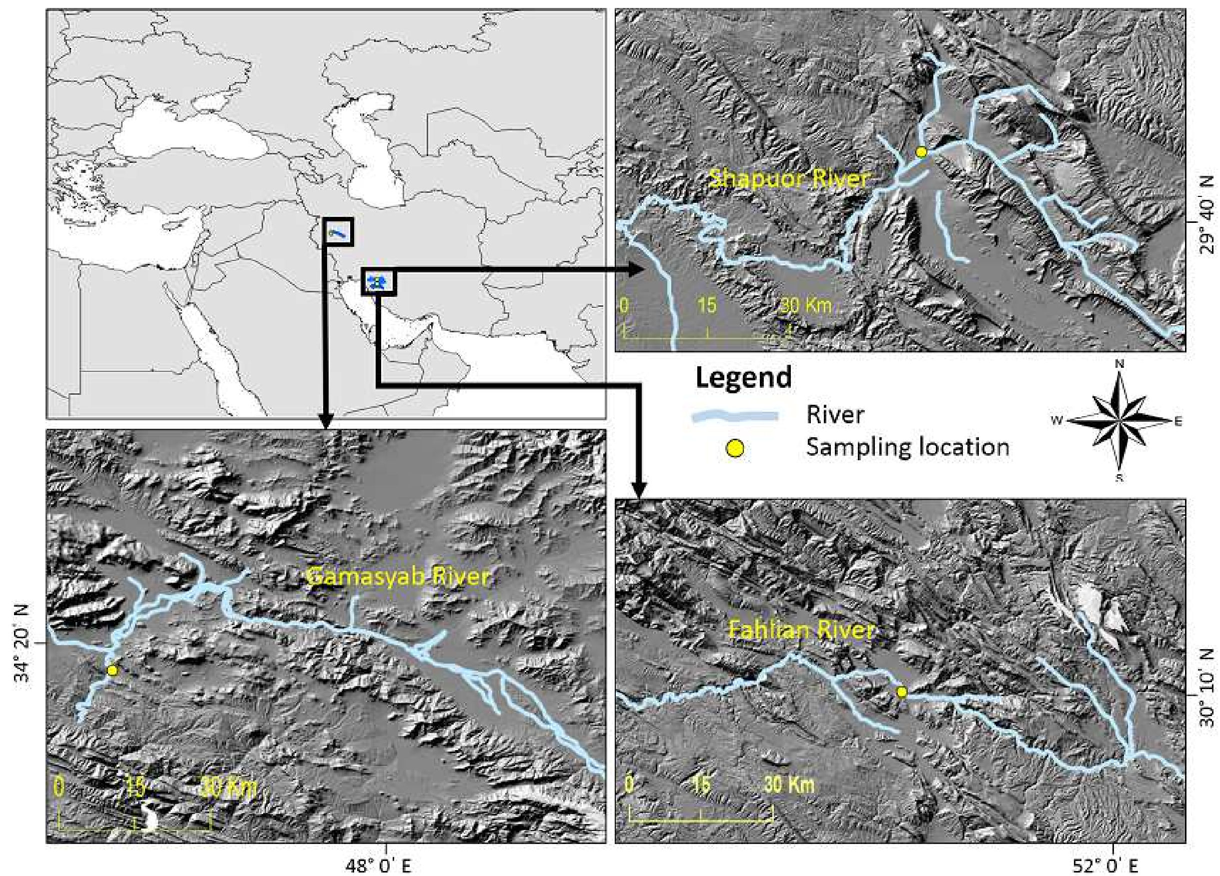

FIGURE 1. Map of the rivers (Gamasyab, Fahlian and Shapour) and sampling locations overlaid on the digital elevation model (DEM)-derived hillshade (80 m resolution) representing the topography of the earth's surface in the west and southwest of Iran.

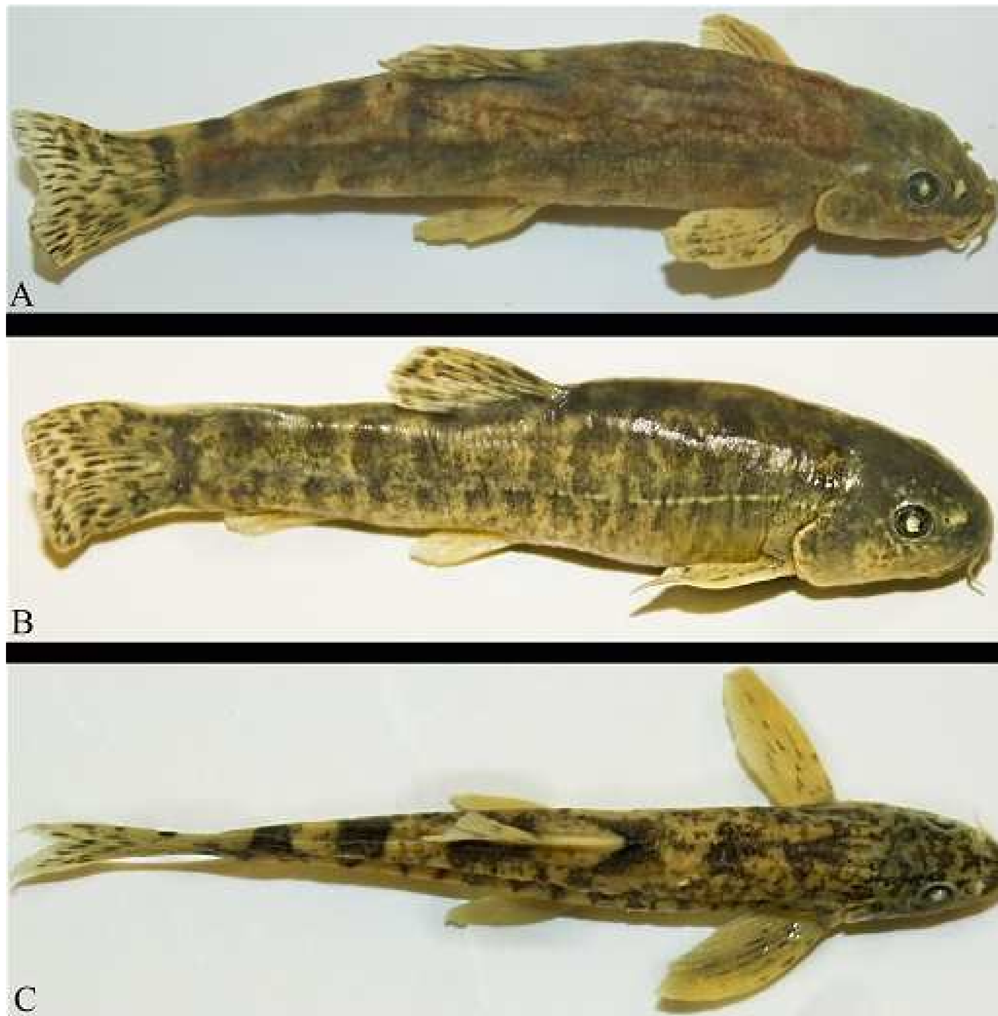

FIGURE 2. Photos showing *Paraschistura bampurensis* (A; Length: 55 mm), *Oxynoemacheilus kiabii* (B; Length: 53 mm) and *Turcinemacheilus saadii* (C; Length: 57 mm); three species of Nemacheilian loaches assessed in this study.

DNA extraction and PCR amplification

Five individuals from three species of Nemachilidae were sequenced, and data were downloaded for further 23 individuals of *Turcinoemacheilae* (Teleostei: Nemacheilidae) from NCBI and EMBL GenBank database (Table 1). No sequence of *O. kiabii* was recognized in GenBanks. Total DNA was extracted from pectoral and pelvic fin using the traditional proteinase-K digestion and standard phenol/chloroform protocol storing at -20°C (Hillis *et al.*, 1996). In order to amplify fragment of mitochondrial COI gene, PCR reactions were conducted using primer cocktails of FishF2-5' TCGACTAATCATAAAGATATCGGCAC3' and FishR2-5'ACTTCAGGGTGACCGAAGAATC AGAA3' (Ward *et al.* 2005). The 25 µl PCR Reaction mixes included 18.75 µl of ultrapure water, 2.25 µl of 10 × PCR buffer, 1.25 µl of$MgCl_2$ (50 mM), 0.25 µl of each primer (0.01 mM), 0.125 µl of each dNTP (0.05mM), 0.625 U of *Taq* polymerase, and 0.5–2.0 µl of DNA template. Amplifications were performed using a Mastercycler® Eppendorf gradient thermal cycler (Brinkmann Instruments, Inc.). The thermal regime consisted of an initial step of 2 min at 95 ˚C followed by 35 cycles of 0.5 min at 94 ˚C, 0.5 min at54 ˚C, and 1 min at 72 ˚C, followed in turn by 10 min at 72 ˚C and then held at 4˚C.

PCR products were visualized on 1.2% agarose gels containing ethidium bromide (10 mg/ml) and the most intense products were selected for sequencing. Products were labeled using BigDye® Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc.) and then sequenced using an ABI 3730 apillary sequencer following manufacturer's instructions. As shown in Table 2, 652 bp of COI consensus barcodes for each species were treated as discrete units to estimate the pairwise level of genetic divergence using K2P correction model (Nei & Kumar, 2000).

TABLE 1. List of specimens included in the analyses, their accession numbers, origin, and source of sequence.

Taxon	Origin	Accession number	Source
Paraschistura bampurensis.	Iran, Shapour River in Fars Province	KP342063	This study
Paraschistura bampurensis.	Iran, Shapour River in Fars Province	KP342064	This study
Paraschistura bampurensis.	Iran, Shapour River in Fars Province	KP342065	This study
Paraschistura bampurensis.	Iran, Shapour River in Fars Province	KP342066	This study
Paraschistura bampurensis.	Iran, Shapour River in Fars Province	KP342067	This study
Oxynoemacheilus kiabii	Iran, Gamasiab River in Kermanshah Province	KP342068	This study
Oxynoemacheilus kiabii	Iran, Gamasiab River in Kermanshah Province	KP342069	This study
Oxynoemacheilus kiabii	Iran, Gamasiab River in Kermanshah Province	KP342070	This study
Oxynoemacheilus kiabii	Iran, Gamasiab River in Kermanshah Province	KP342071	This study
Oxynoemacheilus kiabii	Iran, Gamasiab River in Kermanshah Province	KP342072	This study
Turcinemacheilus saadii	Iran, Gamasiab River in Kermanshah Province	KP342073	This study
Turcinemacheilus saadii	Iran, Gamasiab River in Kermanshah Province	KP342074	This study
Turcinemacheilus kosswigi	Iran Sirvan	KJ179245	Esmaeili *et al*., 2014
Turcinemacheilus kosswigi	Iraq Great Zab	KJ179255	Esmaeili *et al*., 2014
Turcinemacheilus kosswigi	Iran Sirvan	KJ179258	Esmaeili *et al*., 2014
Turcinemacheilus kosswigi	Iraq Little Zab	KJ179260	Esmaeili *et al*, 2014
Turcinemacheilus kosswigi	Iraq Little Zab	KJ179262	Esmaeili *et al*., 2014
Turcinemacheilus kosswigi	Iraq Little Zab	KJ179265	Esmaeili *et al*., 2014
Turcinemacheilus saadii	Iran Karoun	KJ179250	Esmaeili *et al*., 2014
Turcinemacheilus saadii	Iran Karkheh	KJ179253	Esmaeili *et al*., 2014
Turcinemacheilus saadii	Iran Karoun	KJ179257	Esmaeili *et al*. 2014
Turcinemacheilus saadii	Iran Karoun	KJ179248	Esmaeili *et al*. 2014
Turcinemacheilus saadii	Iran Karoun	KJ179261	Esmaeili *et al*., 2014
Turcinemacheilus minimus	Turkey Euphrates	KJ179251	Esmaeili *et al*., 2014
Turcinemacheilus minimus	Turkey Euphrates	KJ179263	Esmaeili *et al*., 2014
Turcinemacheilus minimus	Turkey Euphrates	KJ179249	Esmaeili *et al*., 2014
Turcinemacheilus minimus	Turkey Euphrates	KJ179256	Esmaeili *et al*., 2014
Turcinemacheilus hafezi	Iran Karoun	KJ179259	Esmaeili *et al*., 2014
Turcinemacheilus hafezi	Iran Karoun	KJ179252	Esmaeili *et al*., 2014
Turcinemacheilus hafezi	Iran Karoun	KJ179254	Esmaeili *et al*., 2014
Turcinemacheilus hafezi	Iran Karoun	KJ179264	Esmaeili *et al*., 2014
Paraschistura bampurensis.	Iran Baluchestan	KJ179269	Esmaeili *et al*., 2014
Paraschistura bampurensis.	Iran Baluchestan	KJ179268	Esmaeili *et al*., 2014
Paraschistura malapterura.	Iran Namak	KJ179267	Esmaeili *et al*., 2014
Paraschistura malapterura.	Iran Namak	KJ179266	Esmaeili *et al*., 2014
Turcinemacheilus bahaii	Iran Zayandehroud	KJ179246	Esmaeili *et al*. 2014
Turcinemacheilus bahaii	Iran Zayandehroud	KJ179247	Esmaeili *et al*. 2014

TABLE 2. Estimates of Pairwise Genetic Distances among Nemacheilidae species under Kimura 2-Parameter Model (Kimura, 1980).

E1_P. bampurensis										
E3_P. bampurensis	0.000									
E2_P. bampurensis	0.032	0.032								
E4_P. bampurensis	0.032	0.032	0.000							
E5_P. bampurensis	0.032	0.032	0.000	0.000						
F1_O. kiabii	0.172	0.172	0.167	0.167	0.167					
F2_O. kiabii	0.172	0.172	0.167	0.167	0.167	0.000				
F3_O. kiabii	0.172	0.172	0.167	0.167	0.167	0.000	0.000			
F4_O. kiabii	0.172	0.172	0.167	0.167	0.167	0.000	0.000	0.000		
F5_O. kiabii	0.173	0.173	0.169	0.169	0.169	0.002	0.002	0.002	0.002	
G1_T. saadii	0.158	0.158	0.151	0.151	0.151	0.190	0.190	0.190	0.190	0.192	
G2_T. saadii	0.158	0.158	0.151	0.151	0.151	0.190	0.190	0.190	0.190	0.192	0.002

Data Analysis

The sequences were edited for correction with the SeqScape version 2.6 software (Applied Biosystems), and then submitted to the GenBank Barcode database with the accession numbers KP342063-74. The sequences from GenBank and BOLD databases and also from our dataset were aligned using Mega 5.0 (Tamura *et al.* 2007). Afterwards, pairwise genetic distances were quantified according to Kimura 2-parameter (K2P) distance model (Kimura, 1980) for sequence comparisons. Neighbor-joining (NJ) trees of K2P distances were created to provide a graphic representation of the patterning of divergence among species (Saitou & Nei, 1987). The robustness of the NJ tree was assessed as described by Polgar *et al.* (2017).

RESULTS

The mitochondrial cytochrome oxidase I (COI) region of samples were successfully amplified using PCR. The resulting phenogram of 37 sequences were obtained (Fig. 3). The read lengths were all 652 bp long. According to the Neighbor-joining (NJ) tree (Fig.3), the species in this study were clustered independently. Bootstrap values of species separations were mostly around 100 for *O. kiabii* and *P. bampurensis,* and around 70 for *T. saadii*. NJ tree was consistent in defining the separation among *O. kiabii* and other species, whose clusters were supported by the high bootstrap values (Fig. 3).

Genetic variations were found within the species with a mean K2P distance of 0.006-1.89. Five individuals of *O. kiabii* could not be separated from each other. The K2P distance between species ranged from the least value at 15.36 (*P. bampurensis and T. saadii)* to a maximum value at 19.06% (*O. kiabii and T. saadii*).

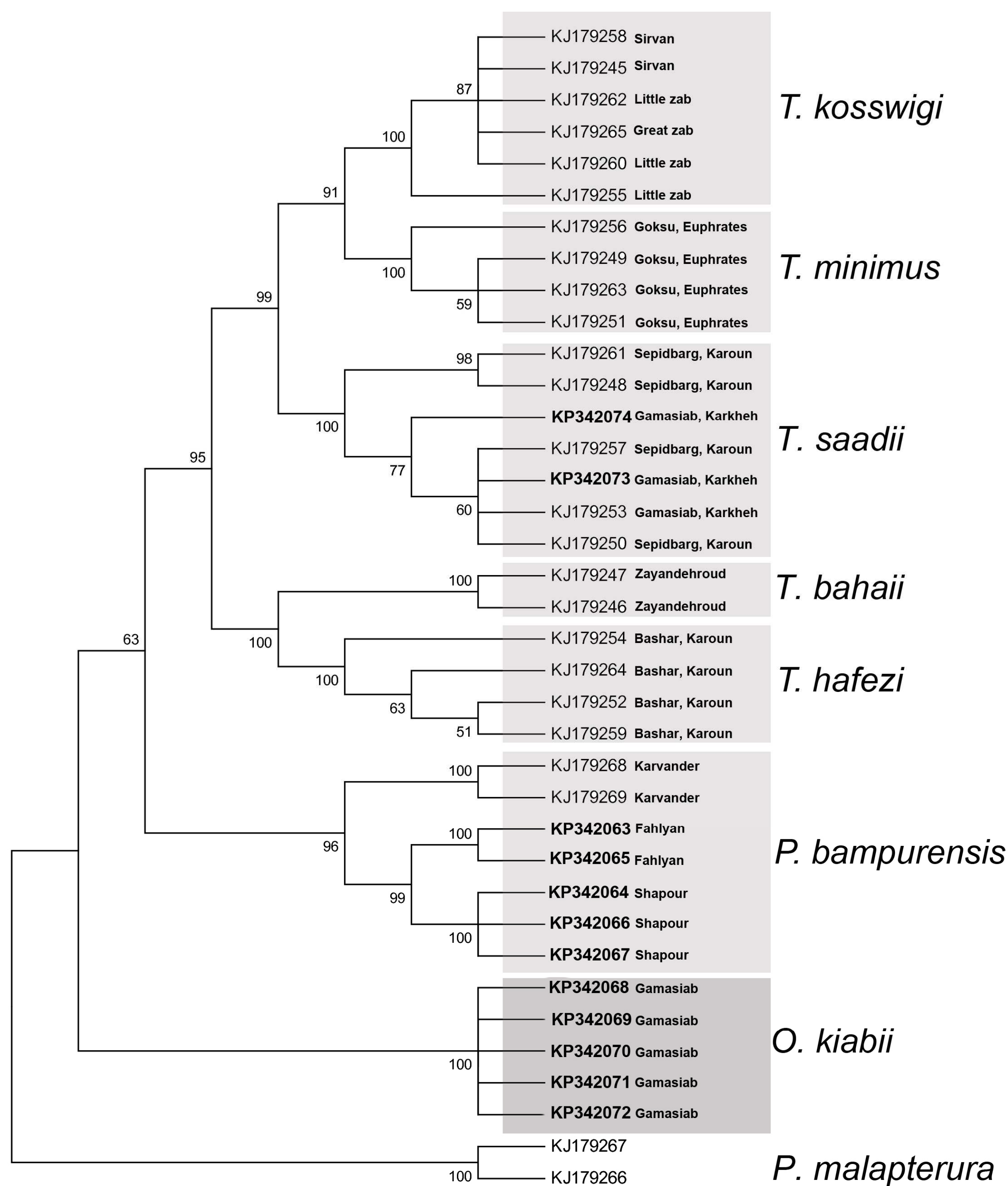

FIGURE 3. Phylogenetic consensus tree of Nemacheilidae species constructed with 652 nucleotide of cytochrome oxidase I (COI) gene using Neighbor-joining (NJ) Method. *Paracobitis malapterura* was used as an out group. Bootstrap values greater than 50 are shown.

DISSCUSSION

This study has validated the efficacy of COI gene for identifying species of Nemachilidae family. Specimens from three species of Nemachilidae were sequenced for the barcode region of COI. With no exception, all samples of the sequenced species were recognized. 100 % of all species was amplified with DNA barcoding primer. Three specimens of *T. saadii* failed to amplify, which could be due to DNA degradation. The phylogenetic tree reconstruction suggested that *Turcinemacheilus minimus* is most closely related to *Turcinemacheilus kosswigi* from Tigris drainage, and *T. minimus* is sister group of *T. kosswigi*. After it had initially been identified as *T. kosswigi* (Breil & Bohlen, 2001), it introduced as a new species, *T. minimus*, from Turkey (Esmaeili *et al.* 2014). Although *Turcinemacheilus bahaii* has been recorded as a new species in Zayanderoud River from Isfahan, Iran and *Turcinemacheilus hafezi* in Bashar River from Yasouj, Iran (Esmaeili *et al.*, 2014), these species are closely related to each other as sister taxa. *T. hafezi* is distributed in Bashar River which is connected to Karoun and Dez Rivers drainages (Golzarianpour *et al.*, 2013), flowing to the deltaic area of Arvand River in Khuzestan province, Iran.

The individuals of *T. saadii*, which were collected from Sepidbarg and Gamasyab Rivers, were separated from each other. According to the phylogenetic tree, Sepidbarg River had more diversity of *T. saadii* in comparison with Gamasyab River. Gamasyab and Sepidberg Rivers flow to Karkheh and Karoun Rivers, respectively, however, both rivers shared the same haplotype. *T. saadii,* which was from Bashar River (a branch of Karoun River), is a sister group of *T. kosswigi* and *T. minimus*. The color pattern of *T. saadii* is unique among *Turcinoemacheilus* in the Middle East region, and therefore, it is clearly distinguishable from *T. kosswigi* and *T. minimus* (Esmaeili *et al.*, 2014). *T. saadii* occurs in sympatry with *T. hafezi,* but these two species are completely separated from each other in phylogenetic tree.

Divergences among individuals of *P. bampurensis* were detected. The individuals of *P. bampurensis* were collected from two different watersheds including three specimens from Shapour River and two specimens from Fahlian River. These specimens of Shapour River were clustered very closely with two specimens of the Fahlian River and far from the other species specimens. Deep divergence among individuals of a species that had been assigned to single species may be a result of the previously unrecognized species or shared haplotype (Ward *et al.*, 2005). The average within species p-distance of *P. bampurensis* was 1.89 % which means that these samples might reflect shared haplotypes. The average within species p-distance of the *P. bampurensis* was remarkably larger than *T. saadii*, so results suggested that two populations of *T. Saadii* and *P. bampurensis* have a great diversity and COI could clearly separate individuals of these populations from each other.

The *Nemeachilus* cladogram (Fig. 3) revealed that *O. kiabii* and *P. bampurensis* are more similar, however, *O. kiabii* is a separate clade. The COI data clearly supported that *O. kiabii,* which has been recorded as a new species in Iran (Golzarianpour *et al.*, 2011a), is different from other *Nemchilous* species with acceptable bootstrap value (100 %). Indeed, there were great genetic divergence among this species and other *Nemachilous* species. Contrary to *P. bampurensis,* other conspecific samples came from the same area, thus we might have somewhat underestimated the extent of within species diversity. It is suggested that sampling should include individuals from different watersheds for freshwater fish as previously mentioned by Ward *et al.* (2005). Confusion in taxonomic assignments as a result of inter specific hybridization (Verspoor & Hammart, 1991) does not probably occur.

Here the usefulness of DNA barcoding has been demonstrated as it has previously been stated that this approach is a powerful tool to identify marine and freshwater fish species from different geographic regions (Hubert *et al.*, 2008; McCusker *et al.*, 2013; Mabragana *et al.*, 2012; Victor *et al.*, 2009; Keskin & Atar, 2013). As we observed, other studies indicated more than 98 % of the analyzed species, especially marine species, could be clearly delimitated through DNA barcoding (Zhang & Hanner, 2012; Costa *et al.*, 2012). Although we observed a high rate of efficiency, in the

case of some freshwater fish species, DNA barcoding has been shown less efficient compared to marine species (April *et al.*, 2011). This is likely due to the fragmentation of rivers and lakes from continental freshwater networks. Such fragmentation may consequently lead to a more pronounced genetic structure among populations and deeper divergence among haplotypes than in the marine realm (Ward *et al.*, 1994). Nonetheless, this study is one of the first steps towards enhancing genetic understanding of relationships among the species of Nemachilidae in Iran, by using a DNA barcoding approach.

In conclusion, this research has successfully identified species of Nemachilidae using some novel and robust molecular techniques. The picture of Nemachilidae is yet incomplete. However, the results from this study have led us to conclude that COI barcodes could be helpful to identify species from the complex family of freshwater fish. The sequences of *O. kiabii* are first genetic data that were submitted to GenBank (KP342068-72). Further genetic or systematic researches should also focus on filling data gaps existing among fish species in water bodies of Iran in order to help establishing a global COI barcode database.

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