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Research Article

Molecular genetic divergence of five genera of cypriniform fish in Iran assessed by DNA barcoding

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Abstract

The present study represents a comprehensive molecular assessment of some family of freshwater fishes in Iran. We analyzed cytochrome oxidase I (COI) sequences for five genus of cypriniform fishes from Iran. The present investigation provides data on genetic structure of some species of Nemachilidae including *Paraschistura bampurensis*, *Oxynoemacheilus kiabii* and *Turcinemacheilus saadii* and Leuciscine cyprinids *Alburnoides bipunctatus* and *Alburnus alburnus* from inland waters of Iran. The DNA sequences of the mitochondrial COI gene in all fishes were amplified, and the resulting sequences were compared to entries in GenBank using the Basic Local Alignment Search Tool for nucleotide data. Individuals were assigned to groups using COI gene sequence divergence analysis which was determined using Kimura 2-parameter distances. Results revealed two major clusters which were inconsistent characterized by mediocre genetic divergence (mean 0.8%). The sequences of *P. bampurensis* showed that specimens collected from four rivers (Beshar, Khersan, Shapour and Fahlian Rivers, southwest of Iran), with 1.89% for Nemacheilian loaches and 0.8% for two Leuciscine cyprinids within-species Kimura two parameters distance, shared haplotype in Neighbor-joining (NJ) tree. Some clusters showed haplotype sharing, or low levels of divergence between species, hindering reliable identification. We discussed the importance of further DNA barcode studies for native and invasive cyprinid species and subsequent submission to GenBank databases for more reliable species match and inference.

Keywords: DNA barcodes, mitochondrial DNA, Leuciscine cyprinids, Nemachilidae, sequence divergence

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Introduction

Fishes are the most species-rich group of vertebrates with about 30,000 species and the family Cyprinidae (Cypriniformes), with about 2,010 species in 210 genera, is the largest family among freshwater fishes (Coad, 2006). According to Esmaeili *et al*. (2018), the most diverse family is the Cyprinidae with 111 confirmed species (43.19%) followed by Nemacheilidae (44 species, 17.12%), Gobiidae (24 species, 9.34%), Cyprinodontidae (14 species, 5.45%), Clupeidae (10 species, 3.89%), Cobitidae (7 species, 2.72%) and Salmonidae (7 species, 2.72%). The genera *Alburnus* and *Alburnoides* are Leuciscine cyprinids found in Europe and northern parts of southwest Asia and encompass 22 species, eight of which can find in Iran (Coad, 2006; Pourkazemi *et al*., 2010). Leuciscine cyprinids species complex, are widespread in temperate and widely distributed in Iran, commonly in small streams and less frequently in the main areas of large river basins of the Caspian Sea, Lake Orumiyeh, Tedzhen River, Kavir, Namak Lake, Zayandeh and Shur Rivers (Esfahan), and Kor River (Kiabi *et al*., 1999; Abdoli, 2000; Nazari *et al*., 2009). Although the taxonomy of Leuciscine cyprinids species (*Alburnoides bipunctatus* and *Alburnus alburnus*) were described (Nazari *et al*., 2011; Seifali *et al*. 2012), genetic structure of these species still has many gaps.

The Nemacheilidae is a species-rich lineage of the order Cypriniforms, consisting mostly of small benthic fishes inhabiting freshwaters of Europe, Asia and Ethiopia and inhabit a variety of Iranian inland waters, e.g. turbulent mountain streams to salty rivers in dry lowlands (Abdoli, 2000, 2011; Golzarianpour *et al*. 2011a). Little is known about the Nemacheilid loaches of western Asia because of their small size and low value for marketing. 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 roaches include numerous species that are morphologically similar. Recent findings regarding Nemacheilidae have led to record of new species in Iran (Golzarianpour *et al*., 2011b, 2013; Esmaeili *et al*., 2013; Freyhof *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 and Shabani, 2013).

Considerable research has addressed the development of molecular markers for identifying fish species (Meyer and Paulay 2005; Roe and Sperling 2007; Zhang and Hanner, 2012) and distinguishing cryptic hybridization (Li *et al*., 2019). Molecular methods and their application for genetic conservation and species differentiation studies have been promoted remarkably

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over the past 20 years (Xiao *et al*., 2009; Mabragana *et al*., 2014; Burghart *et al*., 2014; Jo *et al*., 2014; Nazari, *et al*., 2016; Peoples *et al*., 2017). Various molecular markers, especially markers derived from mitochondrial DNA, have been evaluated for their potential use as DNA barcodes (Amiri *et al*., 2020; Sadeghi *et al*., 2020); such markers include restriction digests or direct sequencing of amplicons from the NADH dehydrogenase 2 gene (Cashner and Bart, 2010), the cytochrome-c oxidase I gene (COI; Ward *et al*. 2005; Kolangi-Miandare *et al*., 2013), or the cytochrome-b gene (Zardoya *et al*. 1999; Herbert and Gregory, 2005; Hajibabaei *et al*., 2007). These molecular markers can provide a simple solution for identification of fish species to develop better management and conservation policies (Lakra *et al*., 2011).

DNA barcoding has known as a reliable molecular tool for species identification. It consists of using a small portion of mitochondrial DNA from a standard agreed-upon position in the genome which can be searched and compared with sequences deposited in databases such as NCBI GenBank and BOLD. Nearly, 655-bp-long fragment from the 5′ region of mitochondrial cytochrome c oxidase subunit I (COI) gene was proposed as the standard region for DNA barcoding of animal species (Hebert *et al*., 2003a,b; Moritz and Cicero, 2004; Hebert and Gregory, 2005; Wong *et al*., 2011; Khoshkholgh and Nazari, 2015). 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 and Chen, 2003; Khoshkholgh and Nazari, 2019).

Considering difficulties in the identification of Nemacheilidae and Leuciscine cyprinid species based on their morphological characteristics, the aim of this study was to provide barcoding data for some species of Nemachilidae and two Leuciscine cyprinid fishes in order to rapid and accurate species identification as well as looking for the internal species marker. In this study, we used DNA barcode methodology to delineate and identify the five genera of the cypriniform fish species from freshwater environments. DNA barcode records generated in this study will be available to researchers for monitoring and conservation of fish diversity in this region.

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Materials and methods

Species sampling

As reference for the extant freshwater fish fauna in Iran, we used the current list of Iranian freshwater fishes (Coad, 2016). The fishes were collected from 5 sampling sites located in Shapour, Fahlian, Gamasyab, Beshar and Zohre Rivers in the west and southwest of Iran. The Beshar River is the most important source of water in the city, which originates from the southeast of Boyer Ahmad and the mountains of the Sepidan and Mamasani in Fars province (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. After joining the other seasonal and permanent streams of routes, the rivers enter to Bouhsher province and finally discharge into the Persian Gulf. 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 roaches were included *Paraschistura bampurensis* from Shapour and Fahlian Rivers, and *Oxynoemacheilus kiabii* and *Turcinemacheilus saadii* from Gamasyab River. The fish were collected by using nets during the May - July, 2012. All specimens were fixed in ethanol (96%) and then transferred to the genetic laboratory at Gorgan University of Agricultural Sciences and Natural Resources for later analysis.

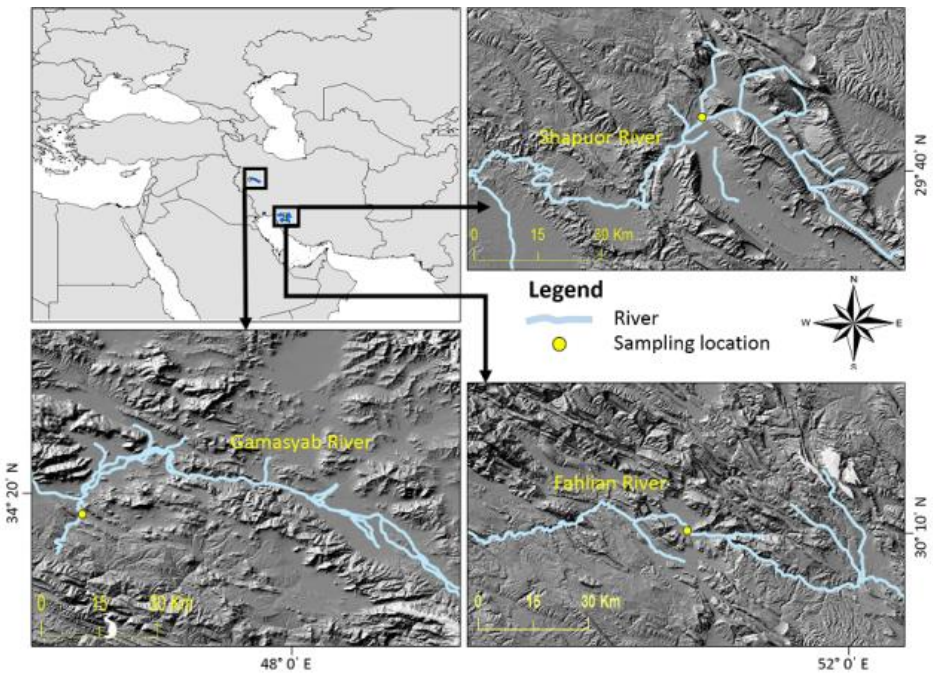

Figure 1: Map of the studied rivers (Gamasyab, Fahlian and Shapour) and sampling locations overlaid on the digital elevation model (DEM)-derived hillshade (80 m resolution) representing the topography of the earth's surface in the west and southwest of Iran.

[DOR: 20.1001.1.15622916.2021.20.3.9.2]

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 databases (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′ TCGACTAATCATAAAGATATCGG CAC3′ 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 at 54˚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 and Kumar, 2000).

Data analysis

Nucleotide sequences were manually aligned, edited, and checked for unexpected stop-codons using the SeqScape 2.6 (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. The COI gene tree was constructed by Neighbour-Joining (NJ) method in Mega 5.0 to provide a graphic representation of the patterning of divergence among the species (Saitou and Nei, 1987). The robustness of the NJ tree was assessed in Mega 5.0 (Tamura *et al*., 2007) by performing bootstrapp analysis with 1000 replications. The number of haplotypes, haplotype diversity, and nucleotide diversity per site for each species were computed by DnaSP v. 6.12.03 software (Rozas *et al*., 2017).

[DOR: 20.1001.1.15622916.2021.20.3.9.2]

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	Tissue
Paraschistura bampurensis.	Iran, Shapour River in Fars Province	KP342064	Tissue
Paraschistura bampurensis.	Iran, Shapour River in Fars Province	KP342065	Tissue
Paraschistura bampurensis.	Iran, Shapour River in Fars Province	KP342066	Tissue
Paraschistura bampurensis.	Iran, Shapour River in Fars Province	KP342067	Tissue
Oxynoemacheilus kiabii	Iran, Gamasiab River in Kermanshah Province	KP342068	Tissue
Oxynoemacheilus kiabii	Iran, Gamasiab River in Kermanshah Province	KP342069	Tissue
Oxynoemacheilus kiabii	Iran, Gamasiab River in Kermanshah Province	KP342070	Tissue
Oxynoemacheilus kiabii	Iran, Gamasiab River in Kermanshah Province	KP342071	Tissue
Oxynoemacheilus kiabii	Iran, Gamasiab River in Kermanshah Province	KP342072	Tissue
Turcinemacheilus saadii	Iran, Gamasiab River in Kermanshah Province	KP342073	Tissue
Turcinemacheilus saadii	Iran, Gamasiab River in Kermanshah Province	KP342074	Tissue
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

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Table 1 continued:

Taxon	Origin	Accession number	Source
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
Alburnus alburnus	Iran, Zagros	HM392001	Tissue
Alburnoides bipunctatus	Iran, Zagros	KJ552440	Tissue
Alburnus mossulensis	Iran, Zagros	29994152	Tissue

Table 2: Estimating 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

Results

The COI region of samples was successfully amplified by using PCR. The resulting phenogram of 37 sequences were obtained (Fig. 2). The read lengths were 652 bp long for all. According to the NJ tree (Fig. 2), 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. 2).

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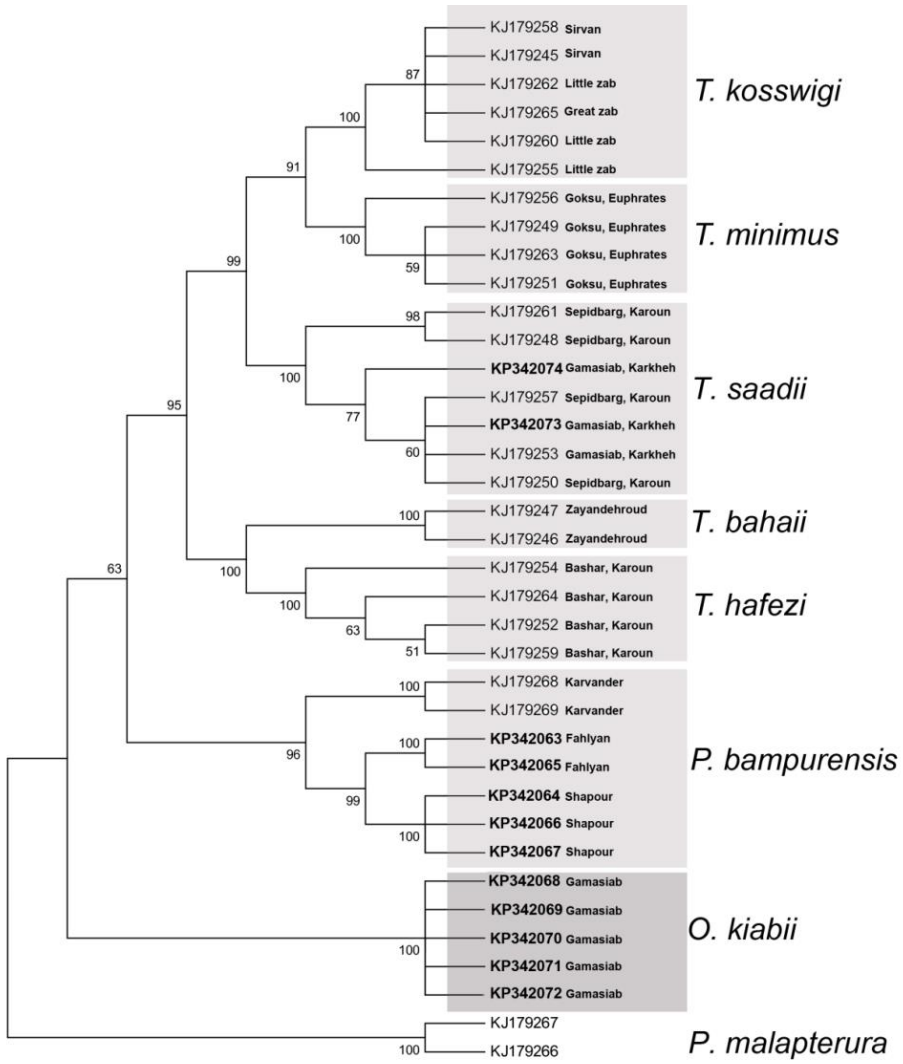

Figure 2: Phylogenetic consensus tree of Nemacheilidae species constructed with 652 nucleotide of cytochrome oxidase I (COI) gene using Neighbor-Joining method. *Paracobitis malapterura* was used as an out group. Bootstrap values greater than 50 are shown.

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*).

The mean genetic distance between *Alburnoides* and *Alburnus* species ranged from 4.1% for *Alburnus alburnus* to 5.8% for *Alburnoides bipunctatus*. The mean genetic distances between *Alburnus alburnus*, *Alburnus mossulensis* and *Alburnoides bipunctatus* species are given in Table 2. Largest mean intraspecific distance was observed in *Alburnoides bipunctatus* (0.58%; Table 3; Fig. 3).

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Table 3: Intraspecific genetic variation of *Alburnoides* and *Alburnus*

Species	N	h± SD	π ± SD	Mean intra-sp ± SE	Max intra-sp	Genetic Distance
Alburnus alburnus	6	0.93 ± 0.08	0.0038 ± 0.0051	0.003 ± 0.012	0.006	0.041 ± 0.025
Alburnus mossulensis	7	0.91 ± 0.04	0.0028 ± 0.0036	0.003 ± 0.005	0.009	0.034 ± 0.011
Alburnoides bipunctatus	6	0.89 ± 0.62	0.0046 ± 0.0014	0.003 ± 0.008	0.003	0.058 ± 0.035

N : sample size; h±SD: haplotype diversity ± standard deviation; π±SD: nucleotide diversity ± standard deviation; Mean intra-sp±SE: mean intraspecies K2P distance±standard error; Max intra-sp: maximum intraspecies K2P distance; Genetic distance±standard error (based on 1000 bootstrap replications).

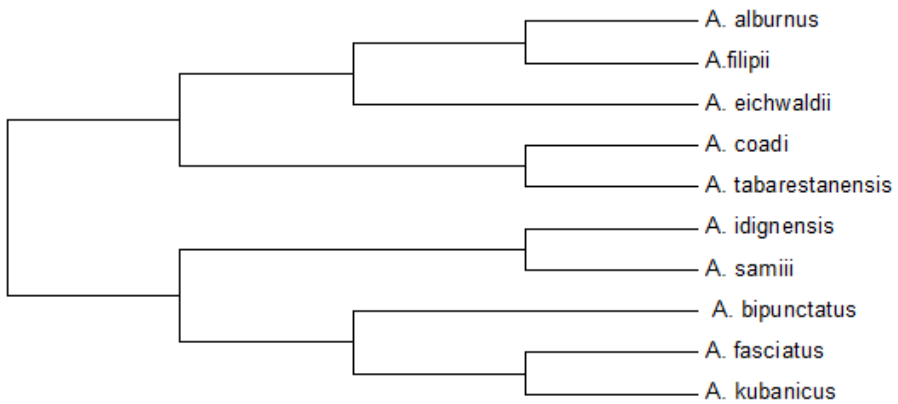

Figure 3: The Neighbour-Joining tree based on COI gene sequences K2P model.

Discussion

In many studies, DNA barcoding approach served as a powerful and reliable tool for the identification of fish species, comprising all major taxa including marine and freshwater species from different geographic regions (Ward *et al*., 2005; Hubert *et al*., 2008; Steinke *et al*., 2009; April *et al*., 2011; Zhang and Hanner, 2012; McCusker *et al*., 2013). According to the objectives of the work, COI sequences were investigated primarily to understand the phylogenetic relationships among the Cyprinid fishes. In this study, specimens from Cyprinid fishes were sequenced for the barcode region of COI. All samples of the sequenced species were recognized with no exception and were amplified with DNA barcoding primer. Three specimens of *T. saadii* failed to amplify, which might be due to DNA degradation. The phylogenetic tree reconstruction suggested that *Turcinemacheilus minimus* is most closely related to *T. kosswigi* from Tigris drainage and *T. minimus* is the sister group of *T. kosswigi*. While it had been initially identified as *T. kosswigi* (Breil and Bohlen, 2001); however it is introduced as a new species of *T. minimus* from Turkey (Esmaeili *et al*., 2014). Although *T. bahaii* has been

[DOR: 20.1001.1.15622916.2021.20.3.9.2]

recorded as a new species in Zayanderoud River from Isfahan, Iran and *T. hafezi* in Bashar River from Yasouj, Iran (Esmaeili *et al*., 2014), these species as sister group are closely related to each other. *Turcinemacheilus hafezi* is distributed in the Bashar River which is connected to Karoun and Dez Rivers drainages (Golzarianpour *et al*., 2013), flowing to the delta area of Arvand River in Khuzestan province, Iran.

The NJ method permitted unambiguous clustering of obtained sequences within groups with high statistical support (85–99%). Phylogenetic analysis of COI data was generally congruent with recent studies of Leuciscine cyprinids (Roudbar *et al*., 2016; Stierandov *et al*., 2016) in determination of closely related species. In the present study, we detected some shared haplotypes between Leuciscine cyprinids. The difference between the causes of the shared haplotypes with the mitochondrial DNA barcoding approach cannot be discriminated. Thus, it is not known whether shared haplotypes represents the natural, ancestral state, or whether the shared haplotypes are already due to human-mediated introgression among lineages. In order to estimate the differences in distance within Leuciscine cyprinids, individuals of the genus *Alburnoides* were analyzed separately to see if three species really have distinct gene pools. So that, they were divided into two groups, according to topology of the built trees. The maximum p-distance between the first and the second groups of Leuciscine species, i.e. *Alburnus alburnus* versus *Alburnus mossulensis*, was noticed to constitute 1.28±0.05%. Within the group 1 (*Alburnoides bipunctatus*) and group 2 (*Alburnus alburnus*), the distances were 1.12±0.03% and 0.094±0.02%, respectively. Thus, COI alone could not be an appropriate marker to infer phylogenetic relationships. Similar results have been obtained by some other researchers who investigated the ability of COI barcode sequences to draw phylogenies (Montagna *et al*., 2016), while the other authors have observed it useful for phylogeny reconstruction (Persis *et al*., 2009).

The *Nemeachilus* phenogram 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 the 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 collected from the same area, thus we might have somewhat underestimated the extent of within species diversity. It is suggested that sampling should be included individuals from different watersheds for freshwater fishes as previously mentioned by Ward *et al*. (2005). Confusion in taxonomic assignments does not probably occur as a result of

[DOR: 20.1001.1.15622916.2021.20.3.9.2]

inter specific hybridization (Verspoor and Hammart, 1991).

In the present study, the application of DNA barcoding has been demonstrated as the powerful tool for identifying marine and freshwater fish species from different geographic regions as it has been stated previously (Hubert *et al*., 2008; McCusker *et al*., 2013; Victor *et al*., 2009; Kim *et al*., 2010; Keskin and Atar, 2013). Likewise, the other studies indicated more than 98% of the analyzed species, especially marine species, could be clearly delimitated through DNA barcoding (Zhang and Hanner, 2012; Costa *et al*., 2012). Although our results showed a high rate of efficiency, DNA barcoding has been shown less efficient compared to marine species in some freshwater fish 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 the populations and deeper divergence among haplotypes than in the marine realm (Ward *et al*., 1994). Nonetheless, this study is one of the first steps of using a DNA barcoding approach to enhance genetic understanding of relationships among the species of Nemachilidae in Iran.

In this study, we utilized standard DNA barcode methodology to delineate between five genus of cypriniform fishes from Iran. The results of the present study indicated that DNA-barcoding is a reliable approach for identification of the five genus of cypriniform fish species from freshwater environments. Cytochrome oxidase I -based DNA barcodes are also useful to uncover intraspecies divergence and to assign potentially new species in the all genus studied.

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