

Morpho-species of the genus *Leuciscus* Cuvier, 1816 (Teleostei: Leuciscinae) from Iran revisited using molecular approaches

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Leuciscus is a genus of fish belonging to subfamily Leuciscinae. While, it is widely distributed throughout Eurasia, our perception of the phylogenetic relationships among its members is mainly based on morphological characters. In this study, two mitochondrial (*CYTB* and *COXI*) and one nuclear (*RAG1*) genomic markers were used in order to assess the taxonomic relationships between two Iranian *Leuciscus* morpho-species. Analysis of the combined dataset using Maximum Likelihood (ML) and Bayesian Inference (BI) resulted in well-resolved trees, where most clades were supported by high statistical values. The trees revealed two clades corresponding with the Caspian Asp, *Leuciscus aspius*, and the Mesopotamian Asp, *L. vorax*. The mean intraspecific and interspecific genetic distance between *Leuciscus* species was 0.5% and 10.6%, respectively. The genetic distance between *L. aspius* sequences of Iran and Europe was 1.1% and the genetic distance between *L. vorax* sequences from Iran and those of Turkey and Syria was 0.8%. This confirmed that the morpho-species *Leuciscus* from the Caspian basin belongs to *L. aspius*, while those from the Tigris basin belong to *L. vorax*. Due to very small genetic distances between some *Leuciscus* members, specific species delimitation analyses are required to clarify genus taxonomy throughout its distribution range.

Keywords: Phylogeny; drainage basin; mitochondrial and nuclear genes; *Aspius*

Introduction

The genus *Leuciscus* Cuvier, 1816 *sensu lato* is the main group of several phylogenetic lineages within Leuciscinae subfamily (Bogutskaya, 1994 & 2002). It cannot be defined by a set of unique traits and characteristics and therefore is not considered as a monophyletic group (Howes, 1984). Using more morphological characteristics (Bianco, 1983; Bănărescu, 1992; Bogutskaya, 1994 & 2002), the members of the *Leuciscus sensu lato* were grouped into three genera namely, *Telestes*, *Squalius* and *Leuciscus*.

The genus *Leuciscus* appeared in Europe in the late Miocene. Until Pleistocene, this highly adaptable genus was widely dispersed throughout Eurasia from the Iberian Peninsula (southwestern Europe) to the Amur River (in China), and from the Kolyma River (southern Siberia) to the Tigris-Euphrates basin (Bănărescu, 1972; Kottelat & Forehoof, 2007). Günther (1899), Saadati (1977), Coad (1980, 1998) and Esmacili et al. (2010, 2018) have reported a number of *Leuciscus* species in Iran including *L. ulanus* Günther,

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1899, *L. gaderanus* Günther, 1899, *L. cephalus* (Linnaeus, 1758), *L. lepidus* (Heckel, 1843), *L. persidis* (Coad, 1981), *L. latus* (Keyserling, 1861), *L. aspius* (Linnaeus, 1758) and *L. vorax* Heckel, 1843. *Aspius aspius* and *A. vorax* were two species which were later attributed to the genus *Leuciscus* by Perea et al. (2010) based on molecular evidences. Finally, three Iranian species were recognised as valid taxa namely, *L. aspius*, *L. vorax* and *L. latus* (Esmaeili et al., 2018; Fricke et al., 2021). The Caspian Asp, *L. aspius*, inhabits the Caspian basin in northern Iran, where it is locally known as “Mashmahi”. The Mesopotamian Asp, *L. vorax*, is found in the Tigris basin in southwest Iran, where it is locally known as “Shelej” (Hashemi et al., 2016; Keivany et al., 2016; Esmaeili et al., 2018). The occurrence of *L. latus* in the Hari River in Iran still needs further confirmation (Saadati, 1977; Esmaeili et al., 2018; Coad, 2020).

So far, various molecular studies have examined the phylogenetic relationship among the members of Leuciscinae subfamily and partly *Leuciscus* using different genomic markers (Durand et al., 2002; Sasaki et al., 2007; Perea et al., 2010; Geiger et al., 2014; Schönhuth et al., 2018). This study was conducted in Iran to reveal phylogenetic patterns among *Leuciscus* members using two mitochondrial and one nuclear genomic marker. It was assessed whether morphologically distinct specimens differ genetically, as well.

Material and Methods

Sampling and identification. A total of 20 fishes were caught using gillnet (mesh size of 5 cm) and a portable electrofishing generator (voltage of 100–120 volts) from different Iranian drainage basins (Figure 1, Table S1) during 2017–2018. In order to carry out molecular analysis, the right pectoral fins of 10 specimens were cut and preserved in 99.7% ethanol. The material was fixed in 10% formaldehyde solution and subsequently deposited in the Zoological Museum, Ferdowsi University of Mashhad (ZMFUM), Mashhad, Iran. To distinguish the morpho-species, the morphological descriptive characters were determined based on Keivany et al. (2016) and Coad (2020). Specimens with silvery body colour, large scales, a thin scaled keel behind the pelvic fins, 65–90 lateral line scales and 7–12 gill rakers were identified as *L. aspius*, while those with silvery-grey or silvery-white body colour, small scales, a relatively wide scaled keel behind the pelvic fins, 82–110 lateral line scales and 9–14 gill rakers were identified as *L. vorax*.

DNA extraction, PCR and sequencing. Total genomic DNA was extracted using salting out method (Aljanabi & Martinez, 1997). The entire mitochondrial cytochrome *b* (*CYTB*, 1140 bp), cytochrome *c* oxidase subunit I (*COXI*, 657 bp) and the nuclear gene including recombination activating gene 1 (*RAG1*, 1473 bp from exon 3) were amplified in a polymerase chain reaction using appropriate primers and conditions as described in Perea et al. (2010).

Data analysis. For all markers, chromatograms were checked and gaps in the sequences were corrected. A total of 84 sequences were used, with 54 downloaded from GenBank and 30 obtained in this study. Three outgroups were selected from the Leuciscidae family: *Squalius lucumonis*, *Chondrostoma nasus* and *Rutilus lacustris* (Table S2). Inter- and intra-specific genetic distances were estimated using ExcaliBAR (Aliabadian et al., 2014). Jmodeltest 2.1.10 tool (Darriba et al., 2012) was used to choose the best-fit evolutionary model. Phylogenetic analyses were performed via Bayesian Inference (BI) and Maximum Likelihood (ML) approaches using MrBayes v.3.2.7 (Huelsenbeck & Ronquist, 2001) and RAxML v.8.2.12 (Stamatakis, 2006) on the CIPRES Science Gateway platform (Miller et al., 2011), respectively.

While nuclear *RAG1* gene sequence was existing in GenBank for some samples only, *CYTB* gene data was available for all samples. As a result, phylogenetic trees for *CYTB*

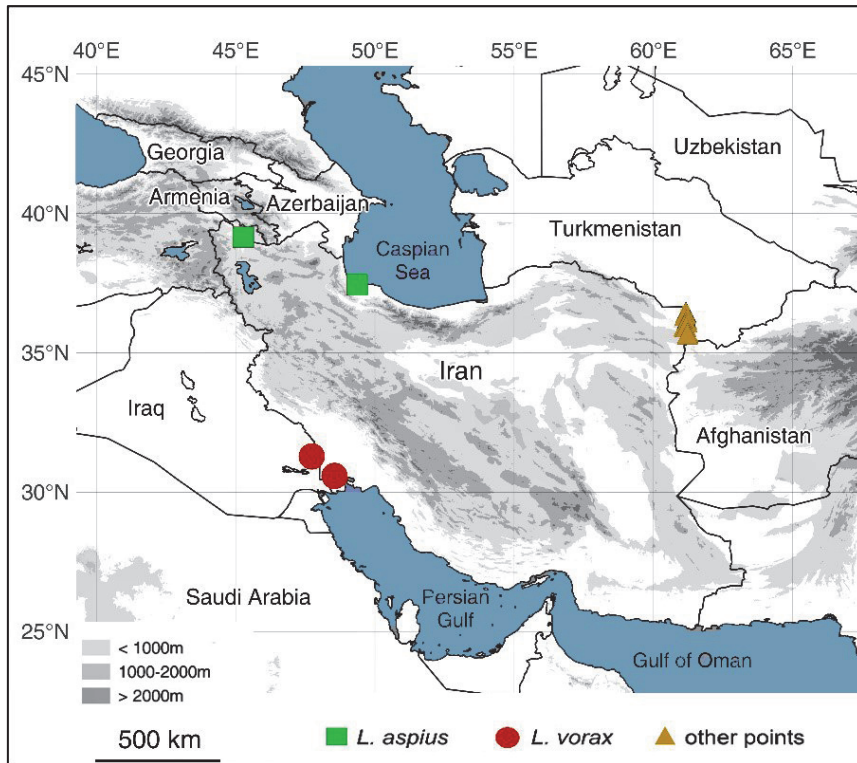


Figure 1. Sampling locations of members of the genus *Leuciscus* in Iran (other point: locations unsuccessfully surveyed for *Leuciscus latus*).

and the combined dataset (*CYTB+COXI+RAG1*: 3275bp) were analysed separately. The ML trees were estimated using bootstrap analysis with 1000 replications and the BI analysis were carried out with 40,000,000 generations using TIM3+G and TrN+I+G models (Bayesian Information Criterion-BIC) for *CYTB* gene and combined dataset, respectively. The first 25% (10 million) generations were excluded as burn-in and remaining trees were considered to compute in each BI analysis. The trees were visualized and compared using Figtree, v.1.4.4, tool (Rambaut, 2018).

Results

The phylogenetic analyses inferred from the combined data set (*CYTB+COXI+RAG1*) (Figure 2) and *CYTB* gene (Figure S1) showed similar and well-supported topologies by both Bayesian and Maximum likelihood inferences. The phylogenetic reconstruction with high support value resulted in four major clades. Clade A comprised of *L. aspius* and *L. waleckii* lineages. Clade B was divided into two subclades: subclade I including *L. idus*, *L. leuciscus*, *L. oxyrrhis* and *L. burdigalensis* lineages and subclade II including *L. latus*, *L. schmidtii* and *L. baicalensis* lineages. The main clades C and D were related to *L. vorax* and *L. merzbacheri* lineages, respectively. Phylogenetic tree analysis further showed that the samples of the present study belong to *L. aspius* and *L. vorax* (*Aspius aspius* and *A. vorax* as per GenBank).

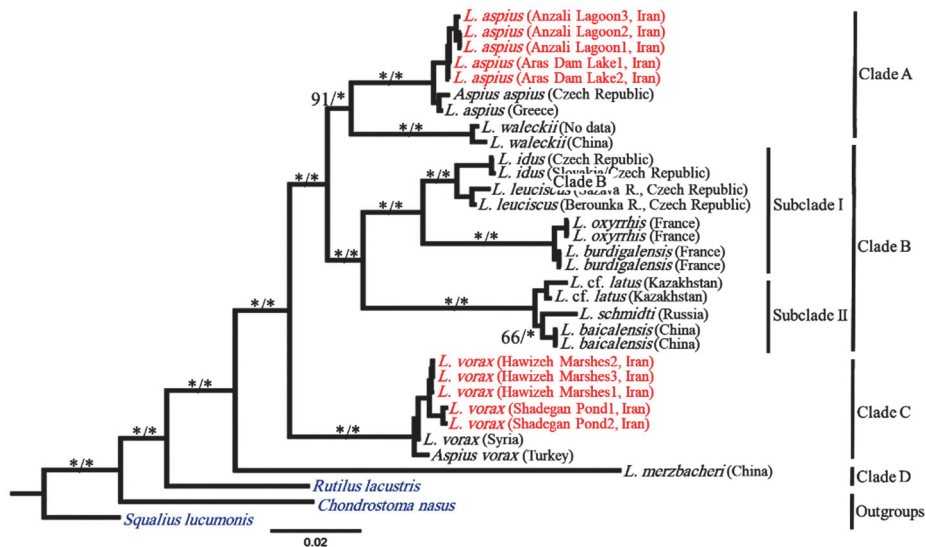


Figure 2. Bayesian Inference (BI) estimate of the phylogenetic relationships among the species included in the study using a combined data set (*CYTB*+*COXI*+*RAG1*) and TrN+I+G model. The numbers at nodes are posterior probability for the BI and bootstrap support for Maximum Likelihood, respectively. * = 100 (posterior probability or bootstrap).

Analysis of the cytochrome oxidase *b* (*CYTB*) gene sequence using Kimura two-parameter (K2p) model measured the mean intraspecific and interspecific genetic distance as 0.3% and 10.6%, respectively. In case of *L. aspilus*, the genetic distance between Iranian samples and GenBank sequences from Greece and Czechia was 1.1%. In case of *L. vorax* species the genetic distance between Iranian samples and those of Syria and Turkey was 0.8% (Table 1). Unexpectedly, within the clade B, a low significant genetic distance was obtained between *L. idus* and *L. leuciscus* lineages, *L. burdigalensis* and *L. oxyrrhis* lineages as well as among *L. baicalensis*, *L. schmidtii* and *L. latus* lineages (Table 1).

Discussion

So far, identification and investigation of the taxonomic status of *Leuciscus* members was carried out based on morphological analyses in Iran. Our results on phylogenetic tree analysis based on two mitochondrial and one nuclear DNA sequences (Figures 2, S1) and the genetic distance values (Table 1) confirmed that the morpho-species *Leuciscus* from the Caspian basin belongs to *L. aspilus*, while those from the Tigris basin belong to *L. vorax*. Cluster analysis based on sequence alignment in GenBank grouped our *L. aspilus* specimen with *Aspius aspilus* from Czechia, and our *L. vorax* specimen with *Aspius vorax* from Turkey and Syria. Our results hereby confirm that *Aspius* should not be regarded as a valid genus (Perea et al., 2010; Geiger et al., 2014; Schönhuth et al., 2018). Due to some reports on occurrence of *L. latus* in Afghanistan (Coad, 2015) and Turkmenistan (Fet & Atamuradov, 2012), the possible presence of this species in Iran can be suggested, as well (Saadati, 1977; Coad, 1980, 1998 & 2020). However, this species was not found

Table 1. Interspecies K2p genetic distance pairwise based on *CYTB* gene (the numbers are by percentage) between the GenBank sequences and the calculated specimens related to the genus *Leuciscus* in the present study. Data on *L. aspius* and *L. vorax* are given in bold.

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	<i>L. aspius</i>													
2	<i>L. aspius</i> (Iran)	1.1												
3	<i>L. baicalensis</i>	10.3	10.4											
4	<i>L. burdigalensis</i>	10.6	10.8	11.2										
5	<i>L. idus</i>	9.7	10.1	10.0	5.8									
6	<i>L. latus</i>	10.3	10.3	1.4	11.6	10.8								
7	<i>L. leuciscus</i>	9.7	9.9	10.0	5.3	1.8	10.3							
8	<i>L. merzbacheri</i>	16.1	16.8	14.5	16.8	16.0	12.7	16.2						
9	<i>L. oxyrrhis</i>	10.4	10.5	11.4	0.4	6.0	11.9	5.5	16.3					
10	<i>L. schmidtii</i>	10.7	11.0	1.2	11.2	10.2	1.2	10.1	14.4	11.4				
11	<i>L.vorax</i>	11.1	11.2	12.6	12.5	12.2	11.2	12.6	15.2	12.4	12.5			
12	<i>L. vorax</i> (Iran)	11.2	11.0	13.0	12.7	12.5	11.5	13.0	15.3	12.6	13.0	0.8		
13	<i>L. waleckii</i>	9.3	9.3	10.9	10.6	10.1	9.9	9.8	14.9	10.6	11.2	12.8	13.1	

in our field surveys and to date there are no reports of the existence of this species in Iran (see Yazdani-Moghaddam et al., 2015; Mousavi-Sabet et al., 2018; Coad, 2020). Therefore, *Leuciscus aspius* and *L. vorax* are the only species of *Leuciscus* genus found in Iran so far.

Supplementary Material

Figure S1 and Tables S1 & S2 are given as a Supplementary Annex, which is available via the “Supplementary” tab on the article’s online page.

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Disclosure Statement

No potential conflict of interest was reported by the authors.

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