

**BRIEF COMMUNICATIONS****Genetic differentiation between two sympatric morphs of the blind Iran cave barb *Iranocypris typhlops***

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The phylogenetic relationship between two sympatric morphotypes of the Iran cave barb *Iranocypris typhlops*, and *Garra rufa*, was investigated by sequencing the cytochrome *c* oxidase I (*col*) region (788 bp) providing the first molecular evidence of their phylogeny. Consistent with their morphological differences, the mean genetic distance between the two forms of *I. typhlops* was significantly higher than generally reported for intraspecific divergence in freshwater fishes. They were phylogenetically closer to *G. rufa* than to any other species.

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The Iranian cave barb *Iranocypris typhlops* Bruun & Kaiser 1944, is a species of ray-finned fish of the family Cyprinidae that was first described by E. W. Kaiser in 1937 (Bruun & Kaiser 1944) from a single locality in the Zagros Mountains, western Iran. More recently, it has been reported in another area (Mahjoorazad & Coad, 2009). *Iranocypris thyphlos* is currently recognized as a vulnerable species according to the IUCN Red Data Book (IUCN, 2010), but the noted report on its new locality may affect its conservation status. Two sympatric forms have been reported within *I. typhlops* (Sargeran *et al.*, 2008) that are morphologically discriminated by the presence or absence of a mouth (mental) disc on the ventral surface of the head, but their phylogenetic status and genetic differentiation are unresolved. The species was suggested to be related to the genus *Barbus* by Bruun & Kaiser (1944), a view that was subsequently rejected by Saadati (1977). More recently, Coad (2011) proposed that the species may be related to the genus *Garra*.

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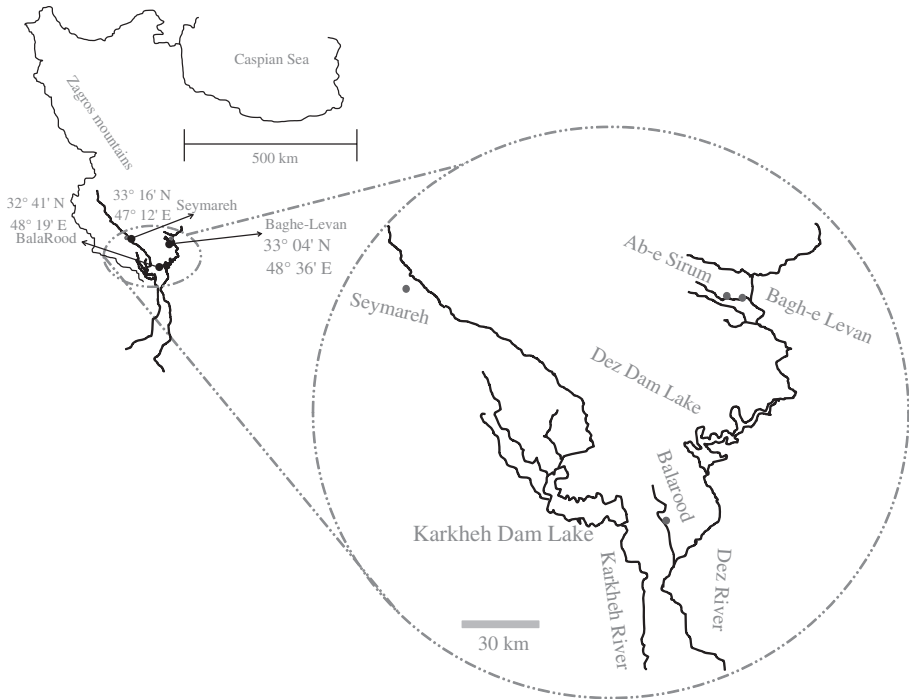


FIG. 1. Location of *Iranocypris typhlops* habitat in Baghe-Levan and the new locality reported by Mahjoorazad & Coad (2009) and the sampling site of the *Garra rufa* specimens (Balarood) in Zagros chain of mountains, Iran.

For this study, partial cytochrome *c* oxidase I (*coI*) sequences of the two *I. typhlops* forms (three specimens of each) collected with a scope net in 2008, and *Garra rufa* (Heckel 1843) (three samples) collected in 2007 were used (Fig. 1). The fish were killed by anaesthetic overdose and fixed in 96% ethanol. Voucher samples encoded as ItBD1-3 (without mental disc), ItD4-6 (with mental disc) and Grb1-3 (*G. rufa* specimens) are held at the University of Shahid Beheshty, Tehran, Iran. The *G. rufa* specimens include only fin clips preserved in ethanol. As the sample of *I. typhlops* was limited to three specimens of each morph, three *G. rufa* specimens were selected to equalize the sample sizes. The *I. typhlops* forms are sympatric and inhabit a well-like pool, the natural outlet of a subterranean limestone system in the Zagros Mountains in the Ab-e Serum Valley near the Tang-e Haft railway station in Lorestan Province, south-west Iran (Bruun & Kaiser 1944) (33° 04' N; 48° 36' E; Fig. 1). *Iranocypris typhlops* has also been reported recently in the Seimareh River system of the Zagros Mountains 131 km from the original location (Fig. 1) (Mahjoorazad & Coad, 2009), but the authors have stated that the specimens are no longer accessible. *Garra rufa* specimens were collected from the Balarood River located in the same basin (Fig. 1).

This study represents the first report of molecular sequences in *I. typhlops* and the *coI* sequence for *G. rufa* from Iran, providing some clarification of their phylogenetic status. It also represents the first test for genetic differentiation between *I. typhlops* forms.

DNA was extracted from fin clips using a Chelex extraction method (Estoup *et al.*, 1996). The *col* gene was amplified using primers FCOI20 (5'-AACCTCTGTCTTC-GGGGCTA-3') and RCOI20 (5'-AGTGGTTATGYGGCTGGCTT-3'), based on the *col* genes for cyprinids available in GenBank. Polymerase chain reaction (PCR) conditions were as follows: a 50 µl final reaction volume containing 5 µl of 10× Taq polymerase buffer, 1 µl of (50 mM) MgCl₂, 1 µl of (10 mM) deoxynucleotide triphosphate (dNTP), 1 µl (10 µM) of each primer, 1 µl of Taq polymerase (5 U µl⁻¹), 7 µl of total DNA and 33 µl of H₂O. Amplification cycles were as follows: denaturation for 10 min at 94° C; 30 cycles at 94° C for 1 min, 50° C for 1 min, 72° C for 1 min and a final extension for 10 min at 72° C. PCR products were sequenced on an ABI-3130xl sequencer using the manufacturer's protocol (Applied Biosystems; www.appliedbiosystems.com).

Sequence data were aligned and processed by using ClustalX (Thompson *et al.*, 1997). A 788 bp 5' *col* amplicon, including a single haplotype for the disc-present form, two haplotypes for the non-disc form and two haplotypes for *G. rufa*, was recovered. The sequences generated were deposited in GenBank under accession numbers JF416296–JF416300.

The haplotypes were compared to published cyprinid sequences using basic local alignment search tool (BLAST) (Altschul *et al.*, 1997) to find similar sequences to be used in the phylogenetic analysis (Table I). To unify the length of the sequences, a common 592 bp length of the selected *col* segment was used. In order to provide a quantitative measure of the sequence distances, and therefore of the divergence among the studied specimens, Kimura two-parameter (K2P) distances (Kimura, 1980) were computed using MEGA4 software (Tamura *et al.*, 2007).

TABLE I. Alphabetical list of cyprinid species included in this study and NCBI-GenBank sequences accession numbers

Species	Accession number	Species	Accession number
<i>Bangana lippus</i>	GU086601.1	<i>G. congoensis</i>	HM418168.1
<i>B. tonkinensis</i>	GU086600.1	<i>G. mirofrontis</i>	ADN94324.1
<i>Barbus balcanicus</i>	HQ600689.1	<i>Garra</i> sp.	JN646099.1
<i>B. barbuis</i>	AB238965.1	<i>Gila conspersa</i>	AP009315.1
<i>B. strumicae</i>	ADT63442.1	<i>G. robusta</i>	DQ536424.1
	ADT63445.1	<i>Hemibarbus barbuis</i>	AB070241.1
<i>Discocheilus wui</i>	GU086599.1	<i>H. labeo</i>	DQ347953.2
<i>D. tetrabarbatus</i>	GU086594.1	<i>Hongshuia microstomatus</i>	GU086586.1
<i>D. yunnanensis</i>	GU086583.1	<i>H. paoli</i>	GU086598.1
<i>D. wuluohensis</i>	GU086607.1	<i>Mylocheilus caurinus</i>	AP010779.1
<i>Garra cryptonemus</i>	GU086570.1	<i>Myxocyprinus asiaticus</i>	NC_006401.1
<i>G. gotyla gotyla</i>	HM798583.1	<i>Pseudocrossocheilus longibullus</i>	GU086571.1
	HM798585.1	<i>P. nigrovittatus</i>	GU086579.1
<i>G. imberba imberba</i>	GU086606.1	<i>P. tridentis</i>	GU086584.1
<i>G. micropulvinus</i>	GU086605.1	<i>P. prochilus</i>	GU086572.1
<i>G. orientalis</i>	HM536884.1	<i>Rectoris posehensis</i>	GU086595.1
<i>G. tengchongensis</i>	GU086604.1	<i>Scaphognathops bandanensis</i>	HM536927.1
<i>G. hughi</i>	HQ219118.1	<i>S. stejnegeri</i>	HM536906.1
<i>G. ornata</i>	HM418163.1	<i>Sinocrossocheilus bamaensis</i>	GU086592.1
	HM418164.1	<i>S. megalophthalmus</i>	GU086593.1

The best-fit model of base substitution was selected based on the Akaike information criterion (AIC) using the online Modeltest programme (Posada & Crandall, 1998). The best model based on AIC values was the transversion model + Γ . Bayesian inference analysis was performed with the software MrBayes 3.1 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). As the selected model could not be run with MrBayes software, the general time reversible model + Γ model (the next best fitting model based on the AIC value), with an α -value of 0.14 was used to analyse the sequences. The Markov Chain Monte-Carlo (MCMC) search was run for 400 000 generations, sampling the Markov chain every 100 generations. The first 25% (1000) trees were discarded as burn-in.

Pronounced *col* sequence divergence between the two forms of *I. typhlops* (3.8% K2P distance) was observed. This sequence divergence is higher than the mean intraspecific divergence values reported in other taxa, *e.g.* 0.46% in Lepidoptera (Hajibabaei *et al.*, 2006), 0.60% in Guyanese bats (Clare *et al.*, 2007), 0.27% in North American birds (Hebert *et al.*, 2004), 0.39% in marine fishes (Ward *et al.*, 2005) and 0.27% K2P distance in freshwater fishes (Hubert *et al.*, 2008). The high sequence divergence between the two forms, along with the morphological differences, including the presence of a mental disc (Fig. 2), the longer intestine, and either a single chambered or bipartite swimbladder in the specimens with disc, *v.* the absence of the mental disc, shorter intestine and bipartite swimbladder in the second form, as reported by Sargeran *et al.* (2008), indicate that the two forms might represent separate species.

Bruun & Kaiser (1948) suggested that *I. typhlops* could belong to the genus *Barbus*. According to the phylogenetic analysis of this study, both *I. typhlops* forms are monophyletic and cluster with *G. rufa*, *Garra gotyla gotyla* (Gray 1830), *Garra orientalis* Nicholas 1925, *Garra tengchongensis* Zhang & Chen 2002, *Garra hughi* Silas 1955 and *Garra* sp. in a group as basal branches, which are paraphyletic with respect to *I. typhlops*. The presence of a mental disc is the key characterization of

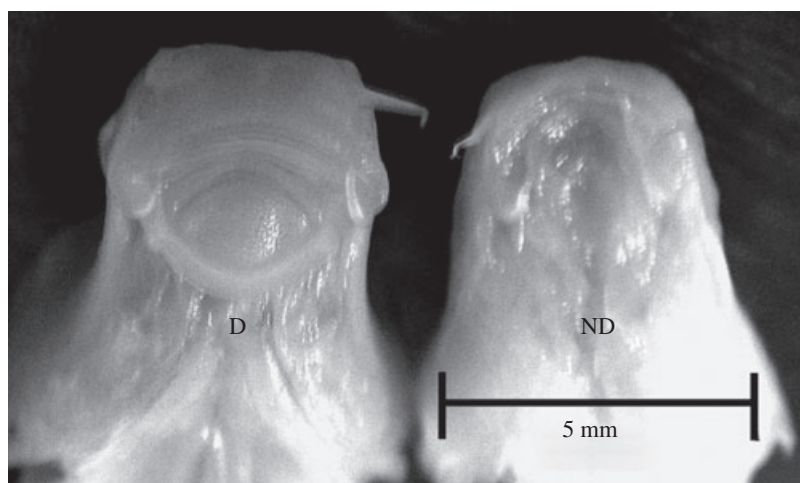


FIG. 2. Ventral view of heads of putative *Iranocypris typhlops*, with (D) and without (ND) a disc. Adapted from Sargeran *et al.* (2008).

species of the subfamily Labeoninae. Within this subfamily, four genera including *Discocheilus*, *Discogobio*, *Garra* and *Placocheilus* are unique in having the lower lip modified into a mental adhesive disc with a posterior margin that is not continuous with the mental region (Zhang, 2005). The genus *Garra* differs from other disc-bearing labeonine genera in having three rows of pharyngeal teeth compared to the two rows in the other three described genera (Zhang, 2005). The three rows of pharyngeal teeth in both *I. typhlops* forms along with a form possessing a mental disc (Fig. 2) can be suggested to indicate a relationship to the genus *Garra*, as is also apparent from the phylogenetic tree (Fig. 3). Conversely, two conclusions can be drawn, based on the observed *coI* sequence divergence and the occurrence of the form lacking the mental disc. If the mental disc is considered to be the key character

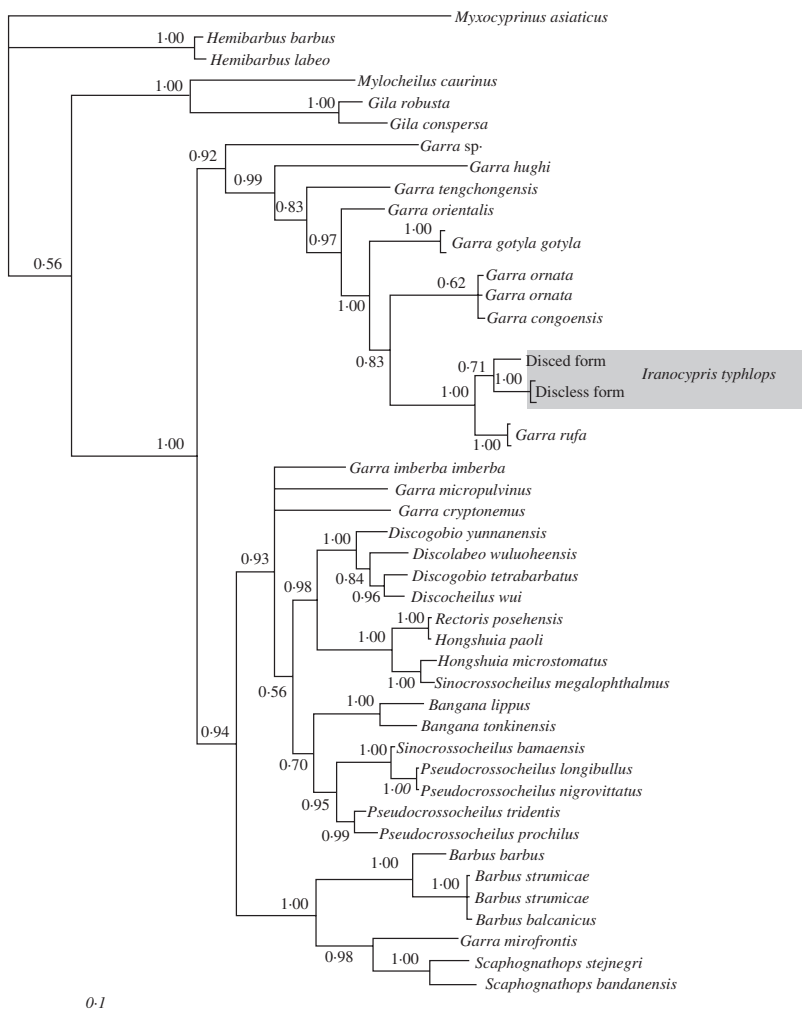


FIG. 3. Phylogenetic tree of *coI* gene sequence inferred by Bayesian approach. The posterior probability values are indicated on branches.

of the subfamily Labeoninae, its absence, along with high sequence divergence of the two forms, might be interpreted as an indication of a different systematic status for the non-disc form. If, conversely, the clustering pattern of the two forms in the phylogenetic tree is considered (Fig. 3), it may be inferred that the absence of the mental disc and other distinguishing morphological characters, such as a shorter intestine, are signs of the evolutionary reduction of some characters in subterranean habitats. Most disc-bearing species are adapted to rapid-water habitats and feed on diatoms, filamentous algae and organic detritus (Zhang, 2005). The absence of the mental disc might be an evolutionary reduction adapting to conditions such as slow-flowing water, or the shorter intestine might be due to the feeding habits of the non-disc form. This could be similar to the lack of eyes in subterranean species. Resolution of this issue awaits further research.

Some species of the genus *Garra*, including *Garra imberba* Garman 1912, *Garra micropulvinus* Zhou, Pan & Kottelat 2005 and *Garra cryptonemus* (Cui & Li 1984) do not cluster in the same group with other congeneric species, indicating that this genus is polyphyletic and requires revision. On the other hand, as *Barbus* species clustered in a highly supported different group, no close phylogenetic relationship among *I. typhlops* and the species of the genus *Barbus* can be inferred based on the phylogram (Fig. 3). Saadati (1977) rejected a relationship among *I. typhlops* and *Barbus* species native to the Tigris River basin based on the size differences among them. These results are similar to the reports on the phylogeny of blind cavefish *Phreatichthys andruzzii* Vinciguerra 1924 and *Garra barreimiae* Fowler & Steinitz 1956 based on *cytb* gene sequence (Colli *et al.*, 2009). These authors found that *P. andruzzii* and *G. barreimiae* are closely related compared to the other cyprinid species included in their study. They reported *P. andruzzii* clustered in a clade that included species of the genus *Garra*. Such results might suggest an evolutionary relationship between *Garra* spp. and blind cavefishes in the studied regions.

The results of this study demonstrated considerable divergence of the two forms of *I. typhlops* and an affinity with *Garra* spp. Research using an integrative approach with larger sample sizes from new localities, additional DNA sequences and morphological characters is recommended to provide more detail regarding the evolutionary relationship between these taxa.

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