



# Sympatric morphotypes of the restricted-range Tashan Cave *Garra*: distinct species or a case of phenotypic plasticity?

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**Abstract** Among Tashan cave barb *Garra tashanensis* inhabiting a small cave in southwest Iran, two mental disc (sucking mouth disc) forms were observed. To assess their phylogenetic relationships, disc-less and disc-bearing individuals were analyzed using mitochondrial cytochrome oxidase subunit I (*COI*) partial DNA sequences. Both mental disc forms nested within one clade with absolute bootstrap support ( $BS=100$ ), and the genetic distances between the disc-bearing and disc-less individuals (0.3–0.8%) were considerably lower than inter-species mtDNA sequence distances reported among members of the genus *Garra*; further, most species delimitation algorithms used here showed disc-bearing and disc-less

Tashan cave barbs to be members of a single taxonomic unit. Hence, the observed mental disc variation was not inferred to be a taxonomic feature or a consequence of character displacement. Instead, it was inferred to be a case of character release to diversify among ecological niches in the limited subterranean habitat or a case of relaxed selection, which should be clarified in detail in follow-up ecological and population genetic studies.

**Keywords** Character displacement · Character release · *Garra tashanensis* · Tashan Cave · Taxonomic feature

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## Introduction

Tashan cave barb *Garra tashanensis* Mousavi-Sabet, Vatandoust, Fatemi and Eagderi 2016, was described from a subterranean habitat with no evident connection to surface habitats (Tashan Cave, Behbahan, Khuzestan Province; lat: 30.851389°N, long: 50.176583°E) in karst formations of southwest Iran (Mousavi-Sabet et al. 2016) in the Marun River drainage. The habitat consists of two small pools hosting an estimated cave barb population of 60–100 individuals, although no conservation listing exists for the species in the IUCN Red List of Threatened Species (<https://www.iucnredlist.org/> accessed 05-Jun-2022). We found two morphotypes of the cave barb within Tashan Cave: one with a well-developed mental/sucking disc—an extension of the lower lip in a number of labeonin fishes including *Garra*—and the other with no mental disc (Fig. 1).

The mental disc has been used as a significant character for taxonomic and systematic inferences within the genus *Garra* (Hashemzadeh Segherloo et al. 2012, 2018; Mousavi-Sabet and Eagderi 2016). Based on mental disc character genetic data, two phylogenetic groups had been considered for the Middle Eastern *Garra* spp., which include the *Garra variabilis* (Heckel, 1843) and *G. rufa* clades (Hashemzadeh Segherloo et al. 2017); the members of the *G. rufa* clade usually have a large highly differentiated mental disc and the members of *G. variabilis* group have a small poorly differentiated mental disc. Although traditionally the presence/absence of the mental disc character in the genus *Garra* was treated as a phylogenetically meaningful character (Menon 1964), the disc character has been reduced independently in different disc-bearing lineages of *Garra* in the Middle East and East Africa (Stiassny and Getahun 2007; Hashemzadeh Segherloo et al. 2017; Levin et al. 2021). The Tashan cave barb is a member of the

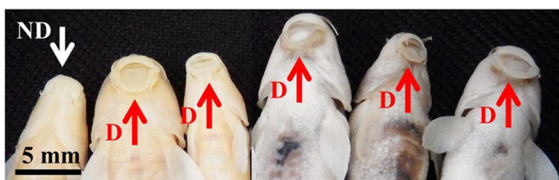
*Garra rufa* (Heckel, 1843) clade of the Iranian *Garra* spp., which is also genetically highly diverged from extant congeners belonging to the same group (the *G. rufa* clade; see Musavi-Sabet et al. 2016 for more details). In the *G. rufa* clade of the Iranian *Garra*, the disc character has been used for description of the Lorestan cave barb *Garra lorestanensis* Mousavi-Sabet & Eagderi, 2016, which is sympatric with the blind Iran cave barb *Garra typhlops* (Bruun & Kaiser, 1944) in the Zagros Mountains of Iran (Sargeran et al. 2008; Musavi-Sabet and Eagderi 2016). This difference in the mental discs of these species was supported by mtDNA (Hashemzadeh Segherloo et al. 2012; Farashi et al. 2014) and genomic data (Hashemzadeh Segherloo et al. 2018). Hence, the mental disc variation which exists in the Tashan cave barb *G. tashanensis* population may also be a phylogenetically and taxonomically important character (character displacement) or alternatively, intra-species morphological diversification (phenotypic plasticity) to exploit a broader ecological niche (character release).

In this study, both mental disc forms from the Tashan Cave are reported for the first time and compared using mitochondrial cytochrome c oxidase subunit I (*COI*) DNA sequences to infer their phylogenetic and taxonomic relationships.

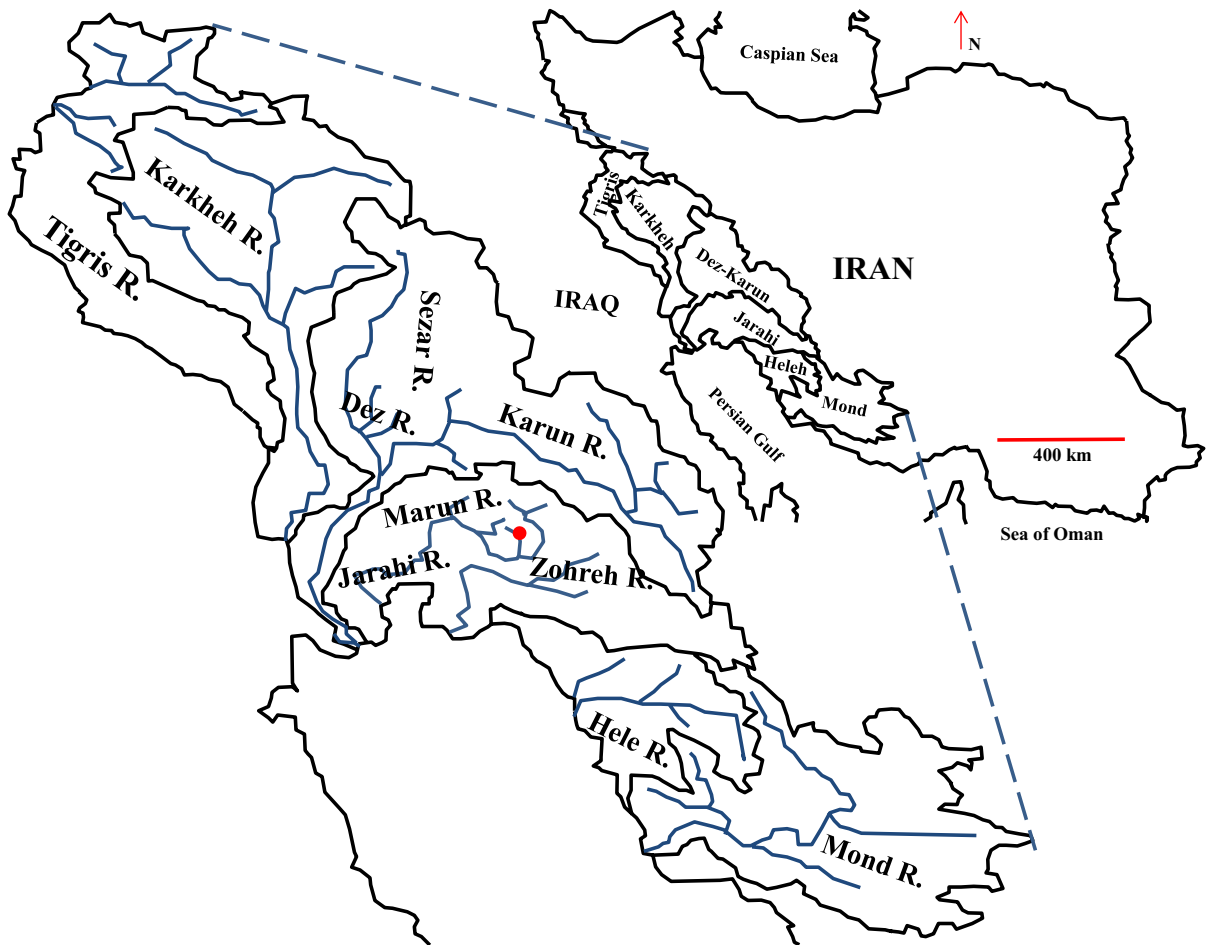
## Material and methods

Due to conservation considerations, we collected seven individuals in February 2018 ( $n=2$ ) and September 2021 ( $n=5$ ) from the Tashan Cave using a dip-net (Fig. 2). The specimens were preserved in 95% ethanol, and after 24 h, the alcohol was refreshed. DNA was extracted using the salt extraction method of Aljanabi and Martinez (1997). Extracted DNA samples were quality-checked via electrophoresis through a 1% agarose gel.

The mitochondrial *COI* region was amplified using the *FishCOI<sub>f</sub>*: 5'-AAYCAYAAAGAYGGYACCCT-3' and *FishCOI<sub>r</sub>*: 5'-CNGGRTGNCRAAGAAYCA-3' primer set (Lara et al. 2010). Each 12.5- $\mu$ l reaction included 6.25  $\mu$ l AccuStart II PCR mix (<http://www.quantabio.com>), 0.5  $\mu$ l of each primer (10  $\mu$ M), 3.25  $\mu$ l deionized water, and 2  $\mu$ l of DNA (10 ng/ $\mu$ l). The thermal profile for amplification included 60 s at 94 °C (preliminary denaturation); 30 cycles at 94 °C (30 s), 55 °C (30 s), and 72 °C (30 s); and one cycle



**Fig. 1** Ventral view of disc-less (white arrow, ND) and disc-bearing (red arrow, D) fish in Tashan Cave



**Fig. 2** Map depicting the location of the Tashan Cave in southwest Iran. The red circle denotes the Tashan Cave

at 72 °C (45 s). DNA sequencing was performed with the *FishCOI*f primer using an ABI Prism 3130 sequencer (<http://www.thermofisher.com>) at the IBIS sequencing platform (Laval University, Quebec City, Canada; <http://www.ibis.ulaval.ca>).

The partial *COI* sequences were edited using BioEdit v. 7.2.5 (Hall 2014). The raw *COI* sequences were aligned using Muscle (Edgar 2004) with the default options in MEGA7 (Kumar et al. 2016). To find similar sequences for phylogenetic analyses, a NCBI BLAST (Johnson et al. 2008) search was performed in the GenBank archive. As some sequences from GenBank did not have the same lengths as our sequences, we used a common 532-bp sequence length for neighbor-joining (NJ) and maximum likelihood (ML) phylogenetic tree reconstructions. However, for haplotype network construction,

a 623-bp sequence length was used, as we included only *G. tashanensis*. The evolutionary distances were computed using the Kimura 2-parameter (K2P) method (Kimura 1980), and the 1st+2nd+3rd codon positions were included. To reconstruct the ML tree, the initial tree(s) for the heuristic search were obtained automatically by applying the neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with the superior log likelihood value. The 1st+2nd+3rd codon positions were included.

To provide support values for each node, 1000 and 500 bootstrap replicates were run for NJ and ML tree reconstructions, respectively. The most appropriate model of nucleotide substitution was selected using jModelTest 2.1.10 (Darriba et al. 2012), based on the

Bayesian information criterion (*BIC*). Other *Garra* spp. sequences from GenBank were used for reconstruction of the phylogenetic tree (Table 1). Three labeonin species—*Bangana ariza* (Hamilton, 1807), *Cirrhinus reba* (Hamilton, 1822), and *Labeo bata* (Hamilton, 1822)—were used as out-groups. The topology of the ML and NJ trees was compared using the R package *ape* (Paradis et al. 2004). To visualize the relationship of haplotypes, a TCS haplotype network (Clement et al. 2002) was constructed using PopART-1.7 (<http://popart.otago.ac.nz>). DNA polymorphism indices were calculated using DNASP v. 6.10.03 (Rozas et al. 2017).

To provide insight into species delimitation patterns, four species delimitation algorithms were applied to assemble species, i.e., by automatic partitioning (ASAP; Puillandre et al. 2021), multi-rate Poisson tree process (mPTP; Kapli et al. 2016), and single and multiple general mixed Yule-coalescent analyses (sGMYC and mGMYC; Pons et al. 2006; Fujisawa & Barraclough 2013). The ASAP analysis was performed on the online platform <https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html> using Jukes-Cantor (JC69), Kimura (K80), and simple distance (*p*-distance) models. For mPTP (<https://mptp.h-its.org/#/tree>) analysis, a ML tree reconstructed using MEGA7 was used as the input tree with the default settings existing on the online server. For sGMYC and mGMYC (<https://species.h-its.org/gmyc/>) analyses, a timetree obtained in MEGA7 was used as the input tree.

## Results

The number of haplotypes (*nH*), haplotype diversity (*Hd*), and nucleotide diversity (*Pi*) calculated for the analyzed individuals (nine individuals: seven in this study (GenBank accession numbers: ON203035–ON203041) and data for two specimens from Musavi-Sabet et al. (2016))

were *nH*=3, *Hd*=0.417, and *Pi*=0.003. The three haplotypes had a *K2P* distance of 0.48–0.80% (1–3 bp difference: three non-synonymous and three synonymous mutations) from one another (Table 2, Fig. 3). Two disc-less individuals and three disc-bearing individuals shared a haplotype with the two disc-bearing individuals reported by Mousavi-Sabet et al. (2016) (Fig. 3). The genetic distances of the analyzed individuals from other congeners from Iran varied from 9.3–10.4% *K2P* distance.

The optimal NJ phylogenetic tree had a total branch length of 0.516. Based on the *BIC*, the model that best described the nucleotide substitution pattern for our data-set was the Tamura-Nei model with a discrete Gamma distribution (5 categories (+*G*, shape parameter=1)) and invariant sites. Based on the comparison made using the *ape* comparePhylo function, both NJ and ML trees were similar in topology except minor differences, which did not affect our inferences. Hence, we present the ML tree with bootstrap support values drawn from both phylogenetic reconstruction approaches (Fig. 3). On both the NJ and ML phylogenetic trees, all the analysed individuals from Tashan Cave nested in a shared phylogenetic group with *G. tashanensis* with absolute bootstrap support (BS=100; Fig. 3). No discrete clustering pattern was observed between the disc-bearing and disc-less individuals. In all the species delimitation models used, except sGMYC, all disc-bearing and disc-less Tashan cave barbs were assigned to a single species (*G. tashanensis*).

## Discussion

### The origin of Tashan Cave barb

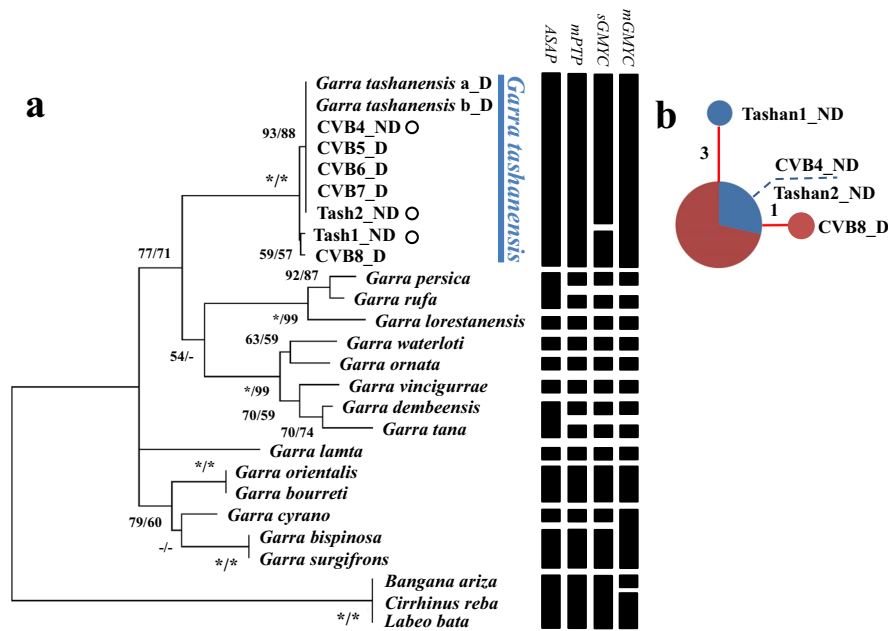
As noted above, Tashan Cave is located in the Marun River drainage, which is adjacent to the Zohreh River

**Table 1** Mitochondrial *COI* sequences obtained from GenBank for use in phylogenetic reconstruction

Accession no	Species	Accession no	Species	Accession no	Species
JX074145.1	<i>Bangana ariza</i>	JF416298.1	<i>Garra lorestanensis</i>	HQ235945.1	<i>Garra tana</i>
MN862482.1	<i>Cirrhinus reba</i>	KX983931.1	<i>Garra orientalis</i>	KY365751.1	<i>Garra tashanensis</i>
MG182184.1	<i>Garra bispinosa</i>	MK074286.1	<i>Garra ornate</i>	KY365751.1	<i>Garra tashanensis</i>
JQ864601.1	<i>Garra bourreti</i>	KM214807.1	<i>Garra persica</i>	MN167175.1	<i>Garra vinciguerrae</i>
MG182191.1	<i>Garra cyrano</i>	MN255080.1	<i>Garra rufa</i>	JX074212.1	<i>Garra waterloti</i>
KF929909.1	<i>Garra dembeensis</i>	KX983934.1	<i>Garra surgifrons</i>	EU030664.1	<i>Labeo bata</i>
MK572210.1	<i>Garra lamta</i>	KXcited in34.1	<i>Garra surgifrons</i>		

**Table 2** Estimates of evolutionary divergence between DNA sequences. Shown below the diagonal is the number of base substitutions per site between given sequences, with standard error estimate(s) above the diagonal. Analyses were conducted using the Kimura 2-parameter model. Codon positions included were 1st + 2nd + 3rd. There were a total of 532 positions in the final dataset

	Tash1_ND1	TASH2_ND2	CVB4_ND3	CVB5_D	CVB6_D	CVB7_D	CVB8_D	G. tashanensis_D	G. tashanensis_D	G. tashanensis_D	G. lorestanensis	G. rufa	G. persica
Tash1_ND1	0.34												
TASH2_ND2	0.76	0.34											
CVB4_ND3	0.76	0.00	0.34										
CVB5_D	0.76	0.00	0.00	0.34									
CVB6_D	0.76	0.00	0.00	0.00	0.34								
CVB7_D	0.76	0.00	0.00	0.00	0.00	0.34							
CVB8_D	0.57	0.57	0.57	0.57	0.57	0.57	0.34						
G. tashanensis_D	0.76	0.00	0.00	0.00	0.00	0.00	0.57	0.30					
G. tashanensis_D	0.76	0.00	0.00	0.00	0.00	0.00	0.57	0.00	0.30				
G. lorestanensis	10.48	10.03	10.03	10.03	10.03	10.03	10.24	10.03	10.03	10.03			
G. rufa	9.80	9.36	9.36	9.36	9.36	9.36	9.56	9.36	9.36	9.36	4.11		
G. persica	9.36	9.36	9.36	9.36	9.36	9.36	9.56	9.36	9.36	9.36	4.52	1.92	



**Fig. 3** **a** Maximum Likelihood (ML) phylogenetic tree reconstructed for a 532-bp *COI* sequence for specimens collected for this study and sequences downloaded from GenBank. Bootstrap support for each branch is denoted beside branches: the values before the slash are the ML bootstrap values and after the slash are NJ bootstrap values. 100% bootstrap values are shown with an asterisk. White-filled circles denote disc-less *G. tashanensis*. Vertical bars indicate species delimitation according different algorithms. **b** TCS haplotype network of *G. tashanensis* sequences (623 bp) produced using

PopART-1.7. Values along the lines between haplotypes indicate the number of mutational differences between each pair of haplotypes. The diameter of pie graphs is proportional to the frequency of haplotypes. Red color denotes disc-bearing individuals and blue color denotes disc-less individuals. The individual codes beside the pie graphs relate to codes of individuals in the phylogenetic tree and identify disc-less individuals with their haplotypes and the disc-bearing individual with a haplotype that differs from the common haplotype

drainage. Hence, the most probable source for colonization of Tashan Cave is the Marun River or its tributaries. As reported in Esmæili et al. (2016), Hashemzadeh Segherloo et al. (2017), and Kiani et al. (2017), the counterparts of *G. tashanensis* in the Marun and Zohreh rivers are *G. rufa*. The high sequence distance between *G. tashanensis* and *G. rufa* or other *Garra* spp. (*K2P* distance > 9%) and the fact that no closely related *Garra* species have been reported in neighboring river drainages refute any possible recent colonization of the cave by the surface-dwelling *Garra* spp. of the region. However, our inference rejecting any recent colonization of surface-dwelling *Garra* spp. to the cave should be confirmed or refuted via the analysis of the nuclear genome, since mtDNA markers would not reflect male-mediated introgression due to maternal inheritance of the mitochondrial genome. It is noteworthy that the Iranian *Garra* spp. have been affected by the invasion of

*G. rufa* during the last glaciation when river drainages in the Persian Gulf basin were connected to the Tigris River (Fagan 2014). For example, *G. mondica* in the Mond River drainage, *G. gymnothorax* in the Karun, Dez, and Karkheh river drainages, and *G. rufa* in the Tigris River drainage that are the modern inhabitants of the surface waters in the Persian Gulf Basin, all show high mtDNA sequence divergence along with high genomic (nDNA) similarity (Hashemzadeh Segherloo et al. 2018). The high mitochondrial divergence along with a high nuclear similarity is probably an indication of the above-noted colonization event and introgression with *G. rufa*. No close relatives of *G. tashanensis* have been reported in surface waters of the Persian Gulf Basin (Hashemzadeh Segherloo et al. 2017, 2018; Kiani et al. 2017). The case of *G. tashanensis* is similar to that of *G. lorestanensis* and *G. typhlops* or *Eidinemacheilus smithi* and *Eidinemacheilus proudlovei* (Freyhof et al. 2016;

Hashemzadeh Segherloo et al. 2016, 2017), for which no closely related surface-dwelling congeners have been reported. This current situation is likely the consequence of ancient extinctions of the ancestral populations via drought periods and later colonization events by species including *G. rufa* and *G. gymnothorax*. In such cases, the subterranean habitats may have proven stable refuges for *Garra* spp. to survive environmental extremes and probably also ecological invasions due to their isolation and stable environmental conditions (Davis et al. 2013). In addition, other mechanisms—including higher rate of natural selection, founder effect, population bottlenecks, and increased rate of fixation of mutations—may have affected the current biogeography of *Garra* spp. in the above-noted region. These should be clarified in follow-up studies using the nuclear genome.

#### Signatures of limited population size

The haplotype diversity in *G. tashanensis* is lower than those reported for *G. lorestanensis* and *G. typhlops* (Hashemzadeh Segherloo et al. 2012; Farashi et al. 2014). Farashi et al. (2014) reported 11 *Cyt-b* haplotypes among 16 *G. lorestanensis* and *G. typhlops* that they analyzed, and Hashemzadeh Segherloo et al. (2012) reported three *COI* haplotypes for six specimens that they studied. In comparison, for nine *G. tashanensis* analyzed in this study and in Mousavi-Sabet et al. (2016), only three *COI* haplotypes are reported. Lower haplotype diversity of *G. tashanensis* than for *G. typhlops* and *G. lorestanensis* may be due to its smaller population size. Bagheri et al. (2016) reported a population size of 300–600 individuals for *G. typhlops* and *G. lorestanensis*, and Mousavi-Sabet et al. (2016) reported a population size of at least 60–100 individuals in the two pools of 20–30 m<sup>2</sup> surface area in Tashan Cave. Compared to the nuclear genome (nDNA), the mitochondrial genome (mtDNA) has an effective size 25% that of the nDNA effective size (Hallerma 2003); this lower effective population size makes mtDNA diversity erode more rapidly in response to random genetic drift or population bottlenecks, which can explain low mitochondrial haplotypic diversity of *G. tashanensis* in Tashan Cave.

#### Character displacement or release?

The existence of two sympatric *Garra* species with differing disc morphologies has been interpreted as a possible case of character displacement (Hashemzadeh

Segherloo et al. 2017; 2021), which allows the respective morphotypes to use different ecological niches and reduce competition in sympatry (Schluter 2000; Gray et al. 2005; Pfennig and Pfennig 2010; Robinson and Pfennig 2013). Our results including phylogenetic and species delimitation analyses do not support the existence of phylogenetically distinct *Garra* species in the Tashan Cave, since there is no important mtDNA differentiation associated with mental disc variation and most likely with speciation (see Zamani-Faradonbe et al. 2021 for more details on inter-species sequence distances among the Iranian *Garra* spp.). It should be noted that Levin et al. (2021) studied sympatric river and stream ecomorphs of *Garra* in the east African White Nile River Basin in Ethiopia using mtDNA (*cyt-b* sequences) and genome-wide SNPs. They did not find any mitochondrial genetic structure among different ecomorphs, but their genomic survey of the same specimens revealed reproductive isolation among different ecomorphs, which was inferred to be a case of recent speciation. In the case of the Tashan cave barb morphotypes, such reproductive isolation may have been developed, which can be clarified in follow-up studies using bi-parentally inherited loci.

At the intraspecific level, when no other closely related species is present in the habitat, it is possible that a number of individuals from the same population undergo morphological changes in their characters to diversify niches available to the species, i.e., character release (Robinson and Wilson 1994). According to the data presented in previous studies on other Iranian subterranean fishes, it appears that mental disc morphology may not always be considered a stable taxonomic character. For example, Hashemzadeh Segherloo et al. (2013) reported an Iran blind cave barb (*G. typhlops*) with a mental disc, while the species previously had been thought to have no mental disc. Our unpublished genomic data show that the disc-bearing *G. typhlops* reported in Hashemzadeh Segherloo et al. (2013) was not a hybrid individual with *G. lorestanensis*, the disc-bearing cave barb sympatric with *G. typhlops*. This indicates variation of the mental disc character within the genus *Garra* and at the intraspecific level. *Garra tashanensis* inhabits a limited subterranean habitat of around 50 m<sup>2</sup> (Mousavi-Sabet et al. 2016) in a semi-arid region where groundwater resources are limited (Ashraf et al. 2021). The Tashan Cave habitat is limited to two small, stagnant pools located 500 m from each other during summer

(Mousavi-Sabet et al. 2016). Mousavi-Sabet et al. (2016) did not report any flowing aquatic habitat in Tashan Cave. The reduction of the sucking disc in the Tashan cave barb can be hypothesized to be related to two mechanisms, which should be tested via further population genetic and ecological studies: (a) Assuming habitat size limitations and other limiting factors that characterize cave habitats, it is possible that the sucking disc variation in *G. tashanensis* is a response to the limited energy resources and limited habitat area. This disc variation increases the likelihood of sustaining a larger population size via resource diversification (Endo and Watanabe 2020) with lower levels of intraspecific competition, i.e., character release. (b) The sucking disc is an adaptation to keep position in fast-flowing habitats (Zhang 2005; Zhou et al. 2005). In Tashan Cave, the habitat is mostly stagnant and hence the reduction of the sucking disc may be a result of relaxed selection, analogous to reduction of eyes in lightless caves (Lahti et al. 2009) or the reduction of sucking disc in *Garra* in limnetic habitats (Stiassny and Getahun 2007). Assuming relaxation of flow-dependent selection that favors its development, the sucking disc should be no longer a fitness-related character and hence has no selective importance in Tashan Cave.

#### Seasonal distribution pattern

Hashemzadeh Segherloo et al. (2018) reported that *G. lorestanensis*, a gular sucking disc-bearing cave barb species, only appears during the fluvial periods of the year when water flows in the habitat, and *G. typhlops*, the disc-less species, is observed during most of the year, independent of water flow regime. The samples in this study were collected in summer and late winter, i.e., in dry and fluvial periods of the year, but no differential frequencies of disc-bearing and disc-less individuals were observed in Tashan Cave; in both sampling periods, we collected both disc-bearing and disc-less individuals. Clarification of factors driving expression of disc-bearing and disc-less morphs in Tashan Cave awaits more-detailed ecological and population genetic studies.

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**Data availability** All sequences and their complementary data related to this study are available under GenBank accession numbers: ON203035-ON203041 and under BOLD TASH project.

#### Declarations

**Ethics approval** The ethical aspects and procedures applied in this research were approved by the education and research board of the Natural Resources and Earth Sciences Faculty, Shahrekord University.

**Conflict of interests** The authors declare no competing interests.

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