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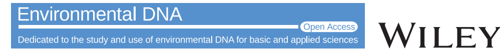

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ORIGINAL ARTICLE

eDNA metabarcoding as a means to assess distribution of subterranean fish communities: Iranian blind cave fishes as a case study

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Abstract

One of the most important steps in conservation of the subterranean life forms is to decipher their distribution and ecology, which is challenging using traditional approaches. Development of an environmental DNA (eDNA) assay provides an efficient means for discovering and monitoring subterranean life forms. In this study, the distribution of three Iranian blind cave fish species (blind Iran cave barb *Garra typhlops*, blind Lorestan cave barb *Garra lorestanensis*, and blind cave loach *Eidinemacheilus smithi*) was assessed using *12S rRNA* eDNA metabarcoding performed using MiFish-U PCR primers and preliminary species distribution modeling (SDM) using bioclimatic data. The majority of sampling localities with positive detection of cave barb eDNA fall within suitable habitats in the Zagros Mountains of Iran. Our results revealed that Lorestan and Iran cave barbs have differential distribution patterns, with some extent of habitat overlap in the vicinity of the originally discovered cave barb locality. According to the observed distribution patterns, the blind Lorestan cave barb and cave loach are mostly distributed in habitats close to the Sezar River (Dez River drainage, Iran), and the blind Iran cave barb is distributed towards the west and probably in a few springs in the Karkheh River drainage. Our data support the previously proposed distribution pattern for the cave barbs, in which the species show partial niche separation and reproductive isolation, with the Lorestan cave barb being a water flow-dependent species and the Iran cave barb being a generalist species preferring variable flow rates. We showed eDNA metabarcoding to be a useful approach for ecological surveys of subterranean fish biodiversity with implications for conservation.

KEYWORDS
distribution, ecology, environmental DNA, habitat use, metabarcoding, species distribution modeling (SDM), subterranean life

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1 | INTRODUCTION

An important step in the conservation of subterranean life is determination of species distribution and ecology (Boyd et al., 2020). In arid and semiarid regions of the world with high anthropogenic exploitation of groundwater (Ashraf et al., 2021), depletion of aquifers can endanger subterranean aquatic life. Unfortunately, during exploitation of aquifers, little attention is paid to subterranean biodiversity, probably due to its cryptic nature. In this regard, clarification of the biology and ecology of the subterranean life can prove an important consideration in managing exploitation of groundwater resources. Data on natural distributions and population sizes can inform conservation planning for different species. Criteria for determining the conservation status of species include restricted geographic range, small population size and decline, and assessed probability of extinction in the wild (see https://www.iucnredlist.org for more details). Species meeting one or more of these criteria can be categorized by the International Union for Conservation of Nature (IUCN) as critically endangered, endangered, or vulnerable.

Collection of data related to population size and distribution can be performed using different visual and physical methods, which have their limitations (Boyd et al., 2020; Wheeler et al., 2004; White et al., 2020). Subterranean habitats intrinsically have limited accessibility, making it difficult and sometimes impossible to study the biology and ecology of associated life forms using conventional methods (Boyd et al., 2020; Danielopol et al., 2000; Deiner et al., 2017).

Among developing methods useful for collecting biodiversity data non-invasively is environmental DNA (eDNA) analysis (Boyd et al., 2020; Deiner et al., 2017; Ficetola et al., 2008; Lacoursière-Roussel et al., 2018). While many studies have utilized eDNA in surface water habitats (e.gAfzali et al., 2021; Deiner et al., 2017; Fujii et al., 2019; Grey et al., 2018; Hinlo et al., 2017; Stoeckle et al., 2017), the application of eDNA to detect subterranean fishes and other subterranean macro-organisms is limited to a few studies, including Sweet Home Alabama cave crayfish *Cambarus speleocoopi* (Boyd et al., 2020), cave salamander *Proteus anguinus* (Gorički et al., 2016, 2017; Vörös et al., 2017), and Australian blind cave eel *Ophisternon candidum* (White et al., 2020).

In the Zagros Mountains of western Iran and eastern Iraq, the occurrence of several different cave fish species—including the blind Iran cave loach (*Eidinemacheilus smithi*; Greenwood, 1976), blind Iraqi Kurdish subterranean loach (*Eidinemacheilus proudlovei*; Freyhof et al., 2016), blind Iran cave barb (*Garra typhlops*; Bruun & Kaiser, 1944), blind Lorestan cave barb (*Garra lorestanensis*; Mousavi-Sabet & Eagderi, 2016), blind Tashan cave barb (*Garra tashanensis*; Mousavi-Sabet et al., 2016), blind Iraq subterranean barb (*Garra widdowsoni*; Trewavas, 1955), and an undescribed species—have been reported (Freyhof et al., 2016; Hashemzadeh Segherloo et al., 2016, 2017, 2018; Mahjoorazad & Coad, 2009; Mousavi-Sabet & Eagderi, 2016; Mousavi-Sabet et al., 2016). These species have been described only from single localities where the aquifer emerges from the ground or from accessible cave habitats. The Iranian blind cave fishes (blind Iran cave barb *G. typhlops*—the species with no mental disc, blind Lorestan cave barb *G. lorestanensis*—the species with a mental disc, and blind cave loach *E. smithi*; hereafter, respectively, called the Iran cave barb, Lorestan cave barb, and cave loach, Figure 1) originally were believed to exist only in a single cave-like karst habitat in the Zagros Mountains in Lorestan Province, west Iran (Figures 2 and 3). This locality is located in the Dez River drainage (close to the Sezar River, a tributary of the Dez River). The neighboring river drainage to the west of the cave barb locality is the Karkheh River drainage (Figure 3). The water level at the cave locality fluctuates during the year, decreasing to a stagnant condition during dry periods of the year (middle spring-late fall, based on our records); a flowing stream

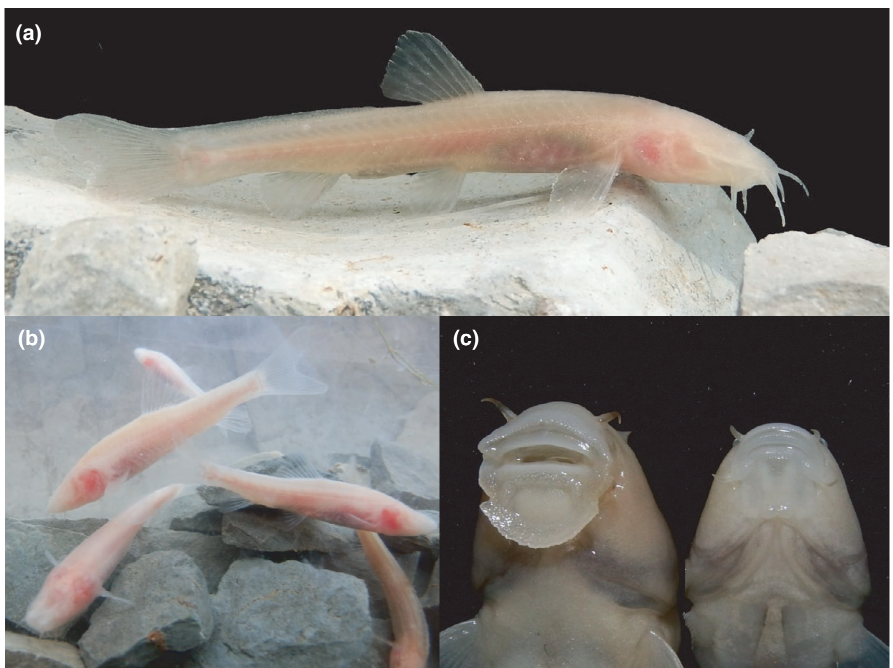

FIGURE 1 Blind Iran cave fishes: (a) cave loach, *Eidinemacheilus smithi*, (b) cave barbs (*Garra lorestanensis* and *Garra typhlops*), and (c) mental (oral) region in *G. lorestanensis* (left, with disc) and *G. typhlops* (right, with no disc)

arises only during fluvial periods of the year. During the fluvial period, water flows out of the cave and after passing a waterfall drains to the Kaye-ru and Sirom streams, repectively, and then to the Sezar River of the Dez River drainage (Figure 2).

There is little information on the distribution and ecology of the Iranian cave fish species in their subterranean habitat, except for undocumented observations and poorly supported hypotheses. Mahjoorazad and Coad (2009) reported a blind subterranean fish (claimed to be Iran cave barb *G. typhlops*) from a locality in the Seimareh River drainage (Karkheh River) 131 km from the cave barb type locality (i.e., the locality where the species were originally described; see Figure 3) in the Dez River drainage. Vatandoust et al. (2019) reported a new locality for the Iran cave fish (*G. typhlops*, *G. lorestanensis*, and *E. smithi*) 31 km from the cave barb type locality (Tuveh Spring, Dez River drainage; Figure 3) and, based on a report by Mahjoorazad and Coad (2009), hypothesized that there may be an aquifer system hosting the blind cave fish with dimensions of 31–162 km. Based on this relatively wide geographical range for the Iran and Lorestan cave barbs, Vatandoust et al. (2019) suggested the need for reconsideration of conservation category of these fishes in the IUCN Red List of threatened species, as one of the criteria used to determine conservation rank of each species is the extent of its range. However, in reports by Mahjoorazad and Coad (2009) and Vatandoust et al. (2019), it was apparently assumed that the

FIGURE 2 Cave barb locality: (a) during fluvial period (late winter-early spring), (b) during dry period, and (c) waterfall located downstream of the cave locality during fluvial period (photos, I. Hashemzadeh Segherloo, Lorestan, Iran)

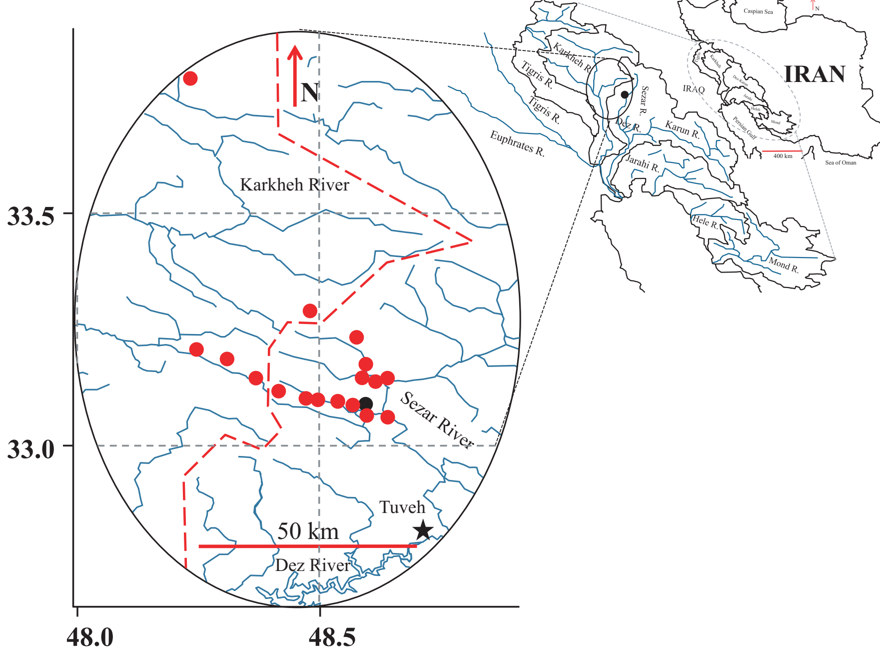

FIGURE 3 Geographic position of the cave barb locality relative to neigboring river drainages. The inset map shows the cave barb locality in the Sezar River in the Dez River drainage. Circles (red and black) denote eDNA sampling localities in the Karkheh River and the Dez River drainages. The black star is the new cave barb locality (Tuveh Spring) reported 31 km from the original cave locality in the Sezar River drainage. The red dashed line separates sampling sites in the Karkheh River and the Dez River drainages

cave barb species in other localities (in the Karkheh River drainage) were also *G. typhlops* or *G. lorestanensis* and that these species had similar distributions within the subterranean habitat. Hashemzadeh Segherloo et al. (2018) reported that, in contrast to the Iran cave barb, which was observed during most of the year, the Lorestan cave barb was mostly captured at the cave barb type locality during the fluvial period (late winter-early spring) when water flows out from the subterranean habitat. This flow-dependent difference in patterns of observation of the Lorestan cave barb may be related to that species having a mental disc, which is believed to be used by labeonin fishes for attaching to the hard substrate and thereby maintaining position in fast-flowing aquatic habitats (Hashemzadeh Segherloo et al., 2017). This can be an indication that the Lorestan cave barb selects mostly flowing-water habitats. In addition to the noted presence or absence of a mental disc, Lorestan cave barb and Iran cave barb are reproductively isolated and have likely evolved via sympatric speciation (Hashemzadeh Segherloo et al., 2018). Based on the presence or absence of mental disc, flow-dependent appearance of Lorestan cave barb in the habitat outflow, and reproductive isolation between Iran and Lorestan cave barbs, we hypothesize that it is possible for these fishes to use different niches in the subterranean habitat with different flow rates and to have different distributions.

No preliminary genome sequence data exist for cave barbs and cave loach. This lack of genetic information, along with the high genetic similarity of cave barbs, hinders our ability to develop species-specific primers for the studied species; hence, toward achieving the goal of testing the mentioned hypothesis on cave barb distribution, we documented patterns of spatial distribution of the Iran and Lorestan cave barbs and cave loach using eDNA metabarcoding of samples from spring waters located between the Sezar River (Dez River drainage) and the Karkheh River drainages in the Zagros Mountains. Indeed, the positive detection of eDNA would provide evidence suggesting that a particular species inhabits this aquifer. Conversely, the absence of eDNA associated with a cave fish species would suggest that this species does not inhabit this aquifer, although false negatives can never be totally ruled out. Testing the hypothesis of differential habitat selection by the Iran and Lorestan cave barbs developed by Hashemzadeh Segherloo et al. (2018), we would expect different patterns of spatial distribution between the respective cave barb species, as the Lorestan cave barb is a specialist species depending on flowing-water habitat patches, and the Iran cave barb is a generalist inhabiting a relatively wide range of flow regimes.

2 | MATERIALS AND METHODS

2.1 | Sampling

The study area is located in the Zagros Mountains (western Iran) between the Karkheh River and the Dez River drainages of the Tigris Basin (Figure 3; Table 1). Prior to sampling, sets of sampling equipment (each including two glass microfiber filters [0.7 μm; Whatman GF/F, 25 mm], a syringe, and its filter holder [60 ml; Becton Dickinson, Franklin Lakes, NJ, USA], two pairs of disposable gloves, plastic forceps, two 1.5- ml cryovials, and a 1 L dark plastic bottle) were sterilized with 10% bleach (0.6 NaClO; not used for filters and gloves) and UV radiation for 30 min. After sterilization of the syringes, bottles, and other plasticware with 10% bleach, they were rinsed with distilled water. Each sampling set was packed in a UV-sterilized plastic bag. Sampling was performed at 20 springs during Summer 2017 over 3 days, localities 1, 3, and 4 on the first day (with two negative field controls), localities 2 and 5–8 (with two negative field controls) on the second day, and localities 9–20 (with three negative field controls) on the third day (see Table 1 for dates). For field negative controls, we filtered 1 L of sterile distilled water before sampling in field as indicated above. One liter of water from the origin of each spring or as close as possible was collected using separate dark plastic bottles. During sampling, disturbing the sediments, which can release eDNA trapped in the sediments, was avoided as much as possible. Water samples were stored on ice in a Styrofoam cooler until filtration. Filtration was performed using piston syringes (see above), which are easily handled in the field with minimal risk of field contamination (Berger et al., 2020; Boivin-Delisle et al., 2021; Leduc et al., 2019). Filters were preserved in 1.5 ml of Longmire's lysis buffer in a 1.5-ml cryotube at 4°C for 3 weeks and then at −20°C before DNA extraction (Lacoursière-Roussel et al., 2018; Longmire et al., 1997).

2.2 | Field data

In addition to our eDNA sampling, we surveyed directly for cavefish species using a dip net in different periods from 2013 to 2016. In addition, 2 h and 20 min of underwater videos were recorded using a GoPro Hero2 and two 4K digital cameras to monitor the relative abundance of the cave loach compared to the cave barbs, since cave loach is a benthic fish and it is not easily viewed and captured physically.

2.3 | DNA extraction and amplification

DNA extraction, polymerase chain reaction (PCR) preparation, and post-PCR procedures were performed in isolation from one another under an ultraviolet-sterilized hood. DNA extraction was performed using the DNeasy® Blood & Tissue Kit according to the manufacturer's instructions (Qiagen), with some modifications (supporting data I). All instruments used in eDNA extraction were bleach and ultraviolet sterilized to avoid contamination. In addition, negative control extractions were performed using distilled water for each extraction set to check for possible contamination. Negative control extractions were treated the same as real samples. To avoid amplicon contamination, pre- and post-PCR operations were carried out in separate rooms with dedicated instrumentation. The amplification of a ~172-bp fragment of the

TABLE 1 Details of sampling localities and presence/absence for different species

Locality name	Locality no	Coordinates		Elevation	Basin	Drainage	Species					Date	Season
		Lat	Long				*G. lorestanensis*	*G. typhlops*	*E. smithi*	*Mastacembelus*	*Capoeta*		
Alashtar-Siahpoosh	1	33.78796	48.23534	1581	Tigris	Karkheh R.	N	N	N	N	Y	2017-07-18	Summer
Ghala-Gol	2	33.28666	48.47898	1821	Tigris	Karkheh R.	N	N	N	N	Y	2017-09-10	
Nozhian waterfall	3	33.23117	48.57498	1363	Tigris	Dez R.	N	N	N	N	N	2017-07-18	
Sarab-e-Nozhian	4	33.23033	48.57748	1371	Tigris	Dez R.	N	N	N	N	N	2017-07-18	
Hokumati	5	33.16636	48.60028	875	Tigris	Dez R.	N	N	N	Y	N	2017-09-10	
Tazan	6	33.14626	48.59034	899	Tigris	Dez R.	N	N	N	N	N	2017-09-10	
Chiler	7	33.13473	48.62104	814	Tigris	Dez R.	N	N	N	N	N	2017-09-10	
Keshvar	8	33.13522	48.64106	760	Tigris	Dez R.	N	N	N	N	N	2017-09-10	
Gargar	9	33.06167	48.64207	661	Tigris	Dez R.	Y	N	Y	N	N	2017-09-10	
Bagh-e-Levan	10	33.07321	48.59685	736	Tigris	Dez R.	N	Y	Y	N	N	2017-09-11	
Cave barb locality	11	33.07724	48.59295	814	Tigris	Dez R.	Y	Y	Y	N	N	2017-09-11	
Naveh	12	33.0775	48.5882	773	Tigris	Dez R.	N	N	N	N	N	2017-09-11	
Ab-Garmeh	13	33.08179	48.57415	830	Tigris	Dez R.	N	Y[a]	N	N	N	2017-09-11	
Sirom	14	33.0967	48.49449	1141	Tigris	Dez R.	N	N	N	N	N	2017-09-11	
Sangshekan	15	33.09851	48.47907	1356	Tigris	Dez R.	N	Y	N	N	N	2017-09-11	
Gardane	16	33.11633	48.41887	2062	Tigris	Dez R.	N	Y	N	N	N	2017-09-11	
Emamzadeh	17	33.13838	48.37123	1866	Tigris	Karkheh R.	N	Y[a]	N	N	N	2017-09-11	
Sarab-Siah	18	33.18044	48.30612	1683	Tigris	Karkheh R.	N	Y[a]	N	N	N	2017-09-11	
Mishanjir1	19	33.19983	48.24449	1508	Tigris	Karkheh R.	N	N	N	N	N	2017-09-11	
Mish-anjir2	20	33.19983	48.24449	1508	Tigris	Karkheh R.	N	N	N	N	N	2017-09-11	

Note: Y: presence; N: absence; yellow highlight: current or near past connection to surface water bodies with no impassable barrier; gray highlight: no direct passable connection to surface water bodies.

[a]Localities with a probability to sample *G. gymnothorax* eDNA.

12S rRNA was performed using the MiFish-U primers (Miya et al., 2015) in a unique dual indexing approach (Berger et al., 2020). Briefly, long forward and reverse primers that contain Illumina flow-cell binding/cluster generation sequences, unique barcodes, Illumina sequencing primer, and MiFish-U sequence-specific sequences were used to generate dual-indexed amplicons in a single PCR reaction that can be sequenced directly on an Illumina MiSeq instrument. The sequences of the oligonucleotides are provided in the supporting information (supporting data II). For each spring's eDNA, five PCR replicates for each eDNA sample and field negative, and a PCR negative run (to confirm the absence of contamination from laboratory procedures) were performed. Each PCR reaction was 50 μl (25 μl Qiagen Multiplex Mastermix, 18-μl deionized water, 2 μl of a 10-μM solution of each primer, and 3 μl of eDNA; Afzali et al., 2021). The amplification reaction was run for one cycle at 95°C for 15 min; 35 cycles at 94°C (30 s), 65°C (90 s), 72°C (60 s); and a final extension at 72°C for 10 min (Berger et al., 2020). The five amplified eDNA replicates for each site were pooled and then were subjected to electrophoresis through a 1.5% agarose gel to check for the quality and size of the amplified fragment. For PCR-negative controls, no fragment was amplified and they were excluded from sequencing because their barcodes were identical to their corresponding samples, but the field negative controls were kept for sequencing. The PCR products then were cleaned up using the Axygen PCR Clean-Up Kit (Axygen). The concentration of each DNA sample was quantified using the AccuClear® Ultra High Sensitivity dsDNA Quantification Kit (www.biotium.com) on a Tecan Spark 10 M Reader (Tecan), after elution in 35 μl of water. The fragment size and concentration of the libraries were determined using an Agilent 2100 Bioanalyzer (Agilent). The libraries then were pooled in equal concentrations to ensure maximum equal sequence depth for each sample and to equalize coverage for all samples (Harris et al., 2010). One run containing 27 samples (20 localities and 7 field controls) was sequenced on an Illumina MiSeq DNA sequencer (Illumina) using MiSeq 600 cycles Reagent Kit V3 (Illumina; sequence length =2 × 300 bp), following the manufacturer's instructions, at the IBIS genomic analysis platform (Université Laval, Québec, Canada: www.ibis.ulaval.ca).

2.4 | Target species *12S rRNA*

As there were no *12S rRNA* sequences of the cave barbs and cave loach in public databases, we produced *12S rRNA* sequences of these species to be used as reference sequences (supporting data III). DNA from fin clips of Iran and Lorestan cave barbs (three specimens of each species) and cave loach (one specimen) was extracted using a salt extraction method (Aljanabi & Martinez, 1997). A ~172-bp fragment of *12S rRNA* was amplified using the primer pairs 12_MIFISHF (5′-GTCGGTAAAACTCGTGCCAGC-3′) and 12s_MIFISHR (5′-CATAGTGGGGTATCTAATCCCAGTTTG-3′) (Miya et al., 2015). Each PCR reaction contained 12.5 μl of Qiagen Multiplex Mastermix (QiaMM), 2 μl each primer solution (10 μM), 5.5 μl of deionized water, and 3 μl of DNA. PCR amplification conditions were an initial denaturation at 95°C for 15 min; followed by 35 cycles of 94°C (30 s), 65°C (90 s), and 72°C (60 s); and a final extension at 72°C for 10 mins. The PCR products were checked by electrophoresis through a 1.5% agarose gel. Sanger sequencing was performed with an ABI 3500 sequencer (Applied Biosystems) at the IBIS genomic analysis platform (Université Laval, Quebec City, Canada, www.ibis.ulaval.ca/) using the forward and reverse primers. Sequences were visually edited using BioEdit v. 7.2.5 (Informer Technologies, 2013).

2.5 | Bioinformatics

The raw data were trimmed for quality using trimmomatic v0.36 (options: LEADING =20, TRAILING =20, SLIDINGWINDOW = 20:20, MINLEN =100, and CROP =200). The trimmed paired-end reads were then merged using flash v. 1.2.11 (options: −*t* = 1, −*z*, −*m* = 30, −*M* = 280). The merged reads were kept if they possessed both forward and reverse amplicon primer sequences using the 03_split_amplicons.sh script from Barque 1.5.2 (https://github.com/enormandeau/barque), an eDNA analysis pipeline that has been shown to be more accurate and efficient alternative to some highly used pipelines for analysing fish eDNA metabarcoding data (Mathon et al., 2021). Chimeras were removed with vsearch v. 2.5.1 (options: --uchime_denovo and --nonchimeras). Reads were then aligned to the 12S mitofish database that was supplemented with the *12S rRNA* sequences of cave fishes using vsearch in Barque (options: --usearch_global, --qmask = none, --dbmask = none, --id =0.97, --maxaccepts =20, --maxrejects =20, --maxhits =20, and --query_cov =0.6). The number of reads per species per site then was summarized into a table using the 07_summarize_results.py script from Barque 1.5.2. During summarization, we excluded sequence reads with low observation frequency of 1–4 times. The eDNA data for the cave barbs and for the cave loach were mapped using DIVA-GIS using the maps available at https://www.diva-gis.org. To assess the phylogenetic resolution of the amplified *12S rRNA* fragment, we reconstructed a Neighbor-Joining (NJ) tree using MEGA7 (Kumar et al., 2016). The options for NJ tree reconstruction were 300 bootstrap replicates, Kimura 2-parameter evolutionary distance, codon positions 1st +2nd +3rd included, and all positions containing gaps and missing data were eliminated. Further, we calculated net nucleotide differences between *12S rRNA* sequences of the cave barbs produced via Sanger sequencing and the sequence reads in each sampling locality, which were assigned to either of the cave barbs with MEGA7. Then, we interpreted only those sequences with no or 1-bp difference within a ~172-bp sequence (over 99% sequence identity) to *12S rRNA* of each cave barb species as sequences belonging to that species. Sequences with over a 1-bp difference from either of the cave barbs were considered as artifacts due to sequencing error.

2.6 | Species distribution modeling

Species distribution modeling (SDM) refers to the application of statistical methods to explore correlation of species distribution and environmental variables to infer ecological niches or potential ranges of geographic distribution (Franklin, 2009; Mammola & Leroy, 2018; Peterson et al., 2011). To model the distributions of the cave fishes and compare the predictions made via SDM with the presence of data obtained via eDNA sampling, we performed SDM using the maximum entropy modeling (Maxent) technique (Bryson et al., 2014; Sánchez-Fernández et al., 2016) as implemented in the Wallace R-based ecological modeling application (Kass et al., 2018). As subterranean fishes show limited distributions and the limited presence records may not be sufficient for SDM, we also used presence records of *G. gymnothorax* in the studied region as a proxy to increase the number of data points for SDM (Johns et al., 2015; Mammola & Leroy, 2018). For the cave barbs, we only used presence records at localities where they have been directly observed. The reason for using *G. gymnothorax* data was its high geographic and phylogenetic proximity to the cave barbs; since at all localities from where cave barbs reported, *G. gymnothorax* inhabits the surface waters (Hashemzadeh Segherloo et al., 2017, 2018). Further, surface-dwelling *Garra* spp. can develop subterranean forms (Kruckenhauser et al., 2011) that can be interpreted as similarity of their thermal physiology and niche to cave barbs (Qiao et al., 2017). Overall, we used 14 presence points for *G. gymnothorax* (11 points) and cave barbs (three points) and excluded the presence points inferred based only on eDNA detection from SDM.

To perform SDM analysis, the presence data (14 localities) were spatially thinned by a distance of 0.5 km with 100 iterations,

TABLE 2 Maxent SDM parameters for *G. gymnothorax* and cave *Garra* spp

RM	FC	AUC_{train}	Average AUC_{test}	Average OR_{MTP}	AICc	Delta AICc	No. of parameters
3	L	0.88	0.77	0.13	289.00	0.00	4
1	L	0.90	0.82	0.13	319.71	30.70	5
2	L	0.88	0.86	0.13	298.77	9.77	5
2	H	0.97	0.79	0.33	NA	NA	13
3	H	0.94	0.79	0.33	299.78	10.78	6
1	H	1.00	0.74	0.40	NA	NA	23
1	LQ	0.91	0.79	0.13	334.68	45.68	6
2	LQ	0.90	0.82	0.13	314.38	25.38	6
3	LQ	0.88	0.84	0.13	295.53	6.53	5
3	LQH	0.95	0.79	0.27	299.56	10.56	6
2	LQH	0.97	0.80	0.33	NA	NA	14
1	LQH	1.00	0.74	0.40	NA	NA	22
3	LQHP	0.95	0.79	0.27	559.56	270.56	11
2	LQHP	0.97	0.80	0.33	NA	NA	15
1	LQHP	1.00	0.74	0.40	NA	NA	28

Note: The parameters are regularization multiplier (RM), feature classes (FC), AUC calculated for training data (AUC_{train}), average AUC calculated for withheld data (average AUC_{test}), average minimum training presence (OR_{MTP}), Akaike information criterion (AICc), difference between each model's AICc and the minimum AICc among all models (delta AICc), and the number of non-zero model coefficients after regularization (no. of parameters). The first row presents the optimal settings.

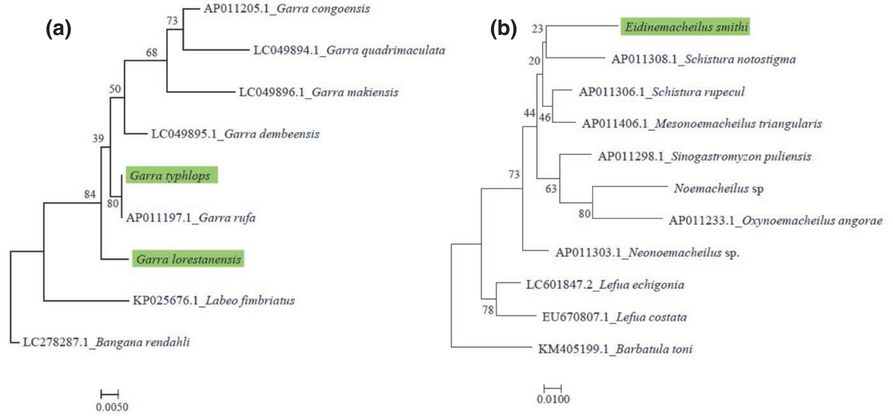

FIGURE 4 Neighbor-Joining tree reconstructed for *12S rRNA* fragment (166 bp) sequenced with forward and reverse MiFish-U primers with 300 bootstrap replicates based on K2P sequence distance considering no gaps. *Labeo fimbriatus* and *Bangana rendahli* were used as out groups. Numbers along branches are bootstrap support values. GenBank accession numbers are given before scientific names in the phylogenetic trees

and the iterations with the maximum number of occurrences were selected. WordClim bioclimatic data were downloaded at a resolution of 30 s to be used as environmental predictors (http://www.worldclim.org). Then, environmental values were extracted at occurrence grid cells using the R package raster, and occurrences with no environmental values were removed. Then, the predictor variables with high collinearity were identified and excluded from modeling using the usdm R package (Naimi, 2015). As we had only presence data, a number of background random points equal to the number of occurrences (14) was taken from the study extent. The occurrence data were partitioned for cross-validation on a random basis into five groups ($K = 5$). To build and evaluate the niche model, we selected the maxent model, which can be performed using the presence-only data (for more details and options used for SDM modeling, see supporting data IV: R code for SDM analysis using maxent model). The feature classes L, LQ, H, LQH, and LQHP were selected, and regularization multipliers were set to 3. To have a measure for efficiency of the model, the area-under-curve statistic (AUC) was calculated (Table 2). The higher the value of AUC (0–1), the higher is the model efficiency at identifying presence or absence at a certain geographic point. The optimum model was selected according to the Akaike information criterion (AICc). After modeling habitat suitability for *G. gymnothorax* and cave barbs, eDNA sampling points were plotted over the habitat suitability map to see whether or not the points fell in localities predicted as suitable for *Garra* spp.

3 | RESULTS

3.1 | Field data

During the period from 2013 to 2016, we performed seven sampling visits to the cave locality, during which six cave loach and 35 cave barbs (15 Lorestan cave barbs and 20 Iran cave barbs) were collected. The limited numbers of cave barbs collected was due to the conservation status of cave fish, difficult sampling conditions, and the limited time (1–2 h) that could be spent each time. During the sampling period, we could collect the Lorestan cave barb only during late winter (March) or early spring (April) when there was an increased water flow rate at the cave barb locality. In the recorded videos, it was not possible to discriminate cave barb species from one another, since they differ only in presence or absence of the mental disc; the morphologically distinct cave loach was recorded only rarely at the cave barb locality (1–2 observations in 2 h of recorded videos).

3.2 | *12S rRNA* resolution

Among six cave barb specimens (three Iran cave barb and three Lorestan cave barb) which were available for sequencing, no intraspecies haplotype diversity was observed, but these two species differed at 2 bp (1.19% sequence divergence) along the 168-bp fragment compared. The amplified fragment does not have a high phylogenetic resolution (Figure 4a) and puts *G. typhlops* with *Garra rufa* (Heckel, 1843) (from GenBank) in a common cluster, although it can discriminate *G. typhlops* from *G. lorestanensis*. This result is contradictory to those of previous genomic, barcoding (*Cytochrome C Oxidase I*; *COI*), or *Cyt-b* studies of cave barbs (Farashi et al., 2014; Hashemzadeh Segherloo et al., 2012, 2017, 2018) that all showed cave barb species to be closer to one another rather than to *G. rufa*, *G. gymnothorax*, or other *Garra* spp. Unfortunately, the origin and other details of the only *G. rufa* complete mitochondrial sequence from which *12S rRNA* sequence was used are not known and may not be reliable (Personal Communication with Jorg Freyhof, Leibniz Institute of Freshwater Ecology and Inland Fisheries). However, *G. rufa* does not occur in the studied region and no *Garra* species other than *G. lorestanensis* and *G. typhlops* have been reported from the habitat (Sargeran et al., 2008; Farashi et al. 2014; Mousavi-Sabet & Eagderi, 2016; Hashemzadeh Segherloo et al., 2012, 2017, 2018). Hence, we assume that DNA sequences assigned to *G. typhlops* in springs with no passable connection to streams can be trusted; otherwise, we treat *G. typhlops* sequences detected in springs with connection to streams cautiously. To resolve these ambiguities, we believe it would be ideal to produce *12S rRNA* Sanger sequences for other species native to surface waters of the studied region to be used as a reference database. In the case of the genus *Eidinemacheilus*, which was described in recent years, there is no problem with phylogenetic resolution of *12S rRNA* sequence, since *E. smithi* does not nest in a common cluster with any closely related genera (Figure 4b), and its maximum identity to other species from GenBank is around 95% (a 9-bp difference along 167 bp of sequence). Such a high phylogenetic divergence between *E. smithi* and other loaches have also been reported using *COI* sequences (Hashemzadeh Segherloo et al., 2016).

3.3 | Environmental DNA

After filtration of the sequence data, a total of 3,862,683 (3,300,092 reads in samples and 562,591 reads in field controls – 508,605 and 52,043 reads in two of the field controls, and a total of 1943 reads in five other field controls, with an average of 388.6 reads) *12s rRNA* sequences were obtained (on average 165,003.9 sequence reads per sample with SD = ±224,217; and 388.6 sequence reads per field control with SD = ±221.08 after excluding the two noted highly contaminated controls). These sequences belonged to 38 genera/species, including 32 genera/species of fishes, five mammals, and one bird species. The taxa detected as caused by contamination were represented by 10 genera/species (supporting data V).

As noted above, the high number of reads in the field negative controls was due to high contamination in two field controls (508,605 and 52,043 reads), but excluding these two field controls that probably had been contaminated, the average read numbers in the remaining five field controls was 388.6 reads. The high

Sequence reads of 38 species/genera detected
1
Non-fish species excluded
Sequence reads of 32 fish species/genera retained
2
Fish species detected in field negatives excluded
Sequence reads of 22 fish species/genera retained
3
Fish species not existing in the region or with less than 10 reads excluded
Sequence reads of 9 fish species/genera retained
4
Exotic fish species existing in the region excluded
Sequence reads of 5 fish species representing four native genera retained

FIGURE 5 Stepwise exclusion of non-target species. Red circles denote the excluded species at each step

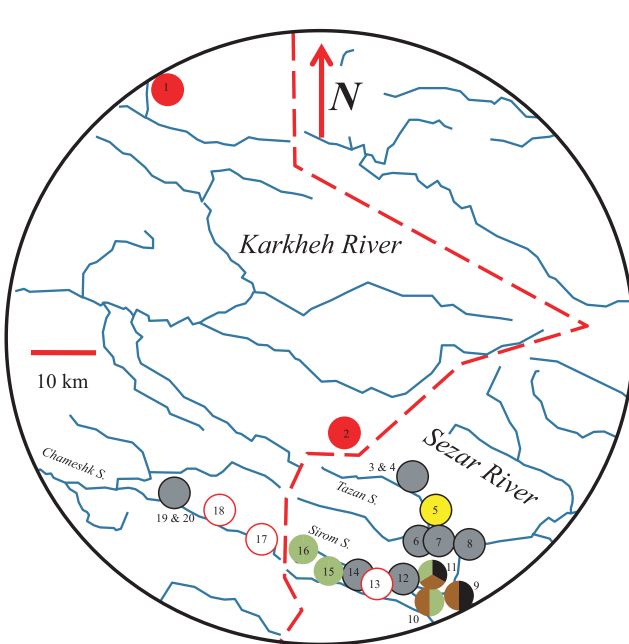

FIGURE 6 Distribution of eDNA (presence/absence) belonging to the cave barbs and cave loach and a few other native fishes (*Mastacembelus* and *Capoeta*). Colors are as follows: brown, cave loach; green, Iran cave barb; black, Lorestan cave barb; white, Iran cave barb (should be confirmed); yellow, *Mastacembelus* sp.; red, *Capoeta* sp.; and gray, no cave or other native fish species

contamination in the two noted field controls probably was because the sampling equipment was used in the Caspian Sea region before sampling in the study area, and apparent failure of the disinfection procedure led to such contamination. Such high contamination would not be related to laboratory procedures, since if high contamination occurred during extraction, we would observe the products on agarose gel. Overall, as the native fishes of interest in this study (Iran and Lorestan cave barbs, and cave loach) were not detected in field controls (only four reads for cave loach and only in one field control – 0.02% of sequence reads in positive-detection sampling sites), we conclude that the results for these species can be trusted. To be conservative, we first deducted this number of reads from the read numbers belonging to cave loach at each sampling site. Thus, after excluding non-fish taxa (six species: one bird and five mammals), fish taxa not existing in the studied basin (*Labeobarbus intermedius*/*Neolissochilus* sp., *Schizothorax* sp., *Rutilus*, *Vimba melanops*, *Sarotherodon* sp., *Astatotilapia* sp., *Cleisthenes* sp., *Esox lucius*, and *Chelon labrosus*), exotic fishes occurring in the region (*Acipenser* spp., *Carassius* spp., *Ctenopharyngodon idella*, and *Oncorhynchus mykiss*) which are farmed species, taxa observed in field negative controls (10 species/genera), and taxa with fewer than 10 reads per site or in total (four species/genera), we identified five native species/genera (Figure 5). These included three known cave fish species (Iran and Lorestan cave barbs, and the cave loach) and two non-cave dwelling fish genera: *Mastacembelus* sp. and *Capoeta* sp. In cases in which sequences were assigned to multiple genera or species (i.e., there were multiple "hits"), only the genus *Capoeta* was native to the study region; hence, we assigned *Capoeta* sp. to such observations. The reason for excluding non-native fishes present in the region was that most of these species were introduced to Iran less than ~60 years ago and more recently to the study region, which is not an enough time for them to develop subterranean forms, or otherwise their eDNA was likely transferred to the spring area by livestock or birds that routinely move overland between streams and springs.

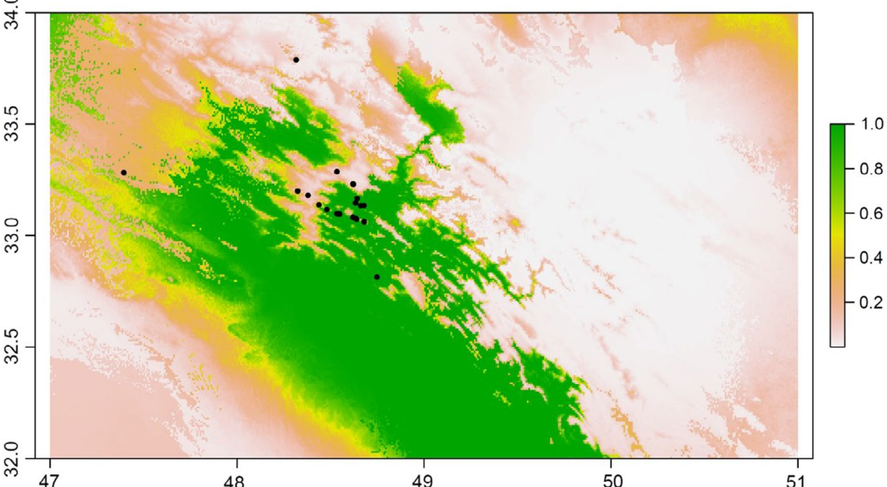

FIGURE 7 Maxent species distribution model (SDM) continous suitability predictions (cloglog transformation) with no treshold on a scale from white (low) to green (high). Black points except the westmost and southmost points are eDNA sampling localities.

Among the sampling localities, only in one spring (cave barb locality: locality 11, Figure 6) was eDNA of both Iran and Lorestan cave barbs and cave loach detected in sympatry. However, in a spring located on the west bank of the Sezar River (Gargar Spring: locality 9), both Lorestan cave barb and cave loach were detected, but Iran cave barb was not detected. In springs located in an upstream tributary of the Sezar River (localities 3–8 in Tazan Stream drainage), no cave barb or cave loach eDNA was detected. In addition, to the west of the cave barb locality (localities 12–20), no cave loach eDNA was detected (Figure 6). With the exceptions of two springs to the west of the cave barb locality (localities 12 and 14 in Sirom Stream of the Sezar River drainage), in localities 13, 15, and 16, eDNA identical to Iran cave barb was detected. In the Karkheh River drainage, two springs (localities 17 and 18) contained eDNA from Iran cave barb, which should be treated cautiously, since *G. gymnothorax* also exists in these localities or streams close to them, and assuming a possible similarity between Iran cave barb and *G. gymnothorax* in *12S rRNA* sequences, there is a probability of misidentification of these species (Figure 6). Additionally, in the Dez River drainage at locality 5, eDNA from *Mastacembelus* sp. was detected, and in the Karkheh River drainage eDNA from *Capoeta* sp. (localities 1 and 2, respectively) was identified (Figure 6).

In localities where eDNA from cave barbs and cave loach co-occurred, the frequency of eDNA from cave loach was much higher than eDNA from cave barbs (supporting data V). All localities in which eDNA from both cave barbs and cave loach was detected and the new locality inhabited by cave barbs and cave loach are located at elevations of 500–700 m above sea level. Compared to the Lorestan cave barb and cave loach, eDNA belonging to the Iran cave barb also was detected at localities with higher elevations.

3.4 | Habitat suitability modeling

Among the bioclimatic variables, six un-correlated predictor variables (Bio3, Bio4, Bio7, Bio12, Bio14, and Bio18) were used for modeling, because the remaining 13 predictor variables showed to be highly correlated (*VIF* >10). We also included the mean annual temperature (Bio1) in the analysis since it was suggested as an important predictor climatic variable affecting subterranean temperature (Badino, 2004; Brookfield et al., 2017). The model showed a positive response to mean annual temperature (Bio1) and mean annual precipitation (Bio12) and a negative response to isothermality (Bio3) and precipitation of driest month (Bio14). Overall, all sampling localities with positive cave barb eDNA detection except one fall within the suitable habitat range (Figure 7). In addition, six sampling points with negative eDNA detection for cave barbs also fall within suitable range detected by SDM.

4 | DISCUSSION

As mentioned in the Results section, at all localities sampled in this study, a number of non-fish and fish species other than the cave fishes were detected, and we excluded them from our discussion for various reasons including: (1) contamination, (2) no reports of the species from the region, (3) limited sequence read numbers (fewer than 10 sequence reads), and (4) occurrence as farmed fishes which may escape to natural water bodies, but whose recent introduction would preclude development of subterranean forms. Hence, to avoid any misinterpretation of the results pertaining to non-target fish species, we focused only on the known cave fish species.

The species not reported in the studied region can be detected due to sequence similarities between other native fishes for which we did not have sequences or unreported introduction from the aquarium or aquaculture industries. The two latter possible sources are likely for tilapia species, since *Astatotilapia* sp. or other closely related species are used as aquarium fishes, and other tilapia species have invaded the lower riches of the drainage and are marketed as edible fish all around the country.

It is possible that cases of apparent contamination were due to failure of the disinfection procedure during field work and would not be related to methods and strategies that we applied for eDNA extraction, PCR amplification, and metabarcoding. Our novel

approach to generate amplicons should minimize the risk of contamination for multiple reasons. First and foremost, the separation of pre- and post-PCR operations into separate rooms with dedicated equipment prevents amplicon contamination of pre-PCR components. Second, there is no need for a second amplification as in most common methods (Miya et al., 2015) or ligation after amplification (Esling et al., 2015), removing an additional source of potential amplicon cross-contamination by laboratory atmosphere. The use of a single PCR reaction for sequence-ready amplicons should also minimize tag jumps that have been suggested to occur mostly in two steps during metabarcoding library preparation: the use of T4 DNA polymerase in ligation-mediated barcoding or as a consequence of chimeras produced during post-ligation PCR (Carøe & Bohmann, 2020; Esling et al., 2015). Since our procedure does not involve the use of a ligation-mediated approach, these major contributors to tag jumps would not be an issue. The disadvantage of using long primers is that they are more expensive because of their length and that a higher number of primers are required to yield high multiplexing capacity, which can increase cost. However, in the long run, they turn out to be more cost and time efficient because they require fewer operations than conventional methods using two consecutive PCRs or those that use adapter ligation.

Among the 20 sites sampled in this study, 14 were located in environmentally suitable ranges predicted by SDM. Eight of these localities had positive detection of cave fish eDNA. In two of these sites, cave fishes have been observed physically, but for six others, this is the first report of the putative presence of cave fishes. The entire studied region is within the karst geological formation of the Zagros Mountains. Regarding SDM results, it is not surprising to have detected cave barb eDNA across a wider range than previously known, since subterranean fishes can move long distances in subterranean karst networks (Vatandoust et al., 2019); otherwise their eDNA can be transported long distances in karst systems due to the absence of degrading environmental factors like ultraviolet radiation and long-distance transport of eDNA in flowing water (Barnes & Turner, 2016; Deiner & Altermatt, 2014; Gorički et al., 2017). Another factor potentially explaining the detection of eDNA in new localities is the transport of eDNA by predators (Thomsen & Willerslev, 2015), which is unlikely for our case, since we did not detect any eDNA belonging to carnivores (mammal or bird; MiFish primers can detect eDNA from those vertebrates) and in most positive detection cases, we had collected eDNA samples only from water emerging at the origin of springs (four springs at localities 10, 13, 17, and 18 were directly connected to streams and such detections should be treated cautiously). Based on a BLAST search in GenBank, there was no difference between *G. typhlops* and the available *G. rufa 12S rRNA* amplicon sequences. *Garra rufa* does not exist in the studied region, but *G. gymnothorax*, for which there are no *12S rRNA* sequence data, is widely distributed in surface waters in the region. There is no reported subterranean form of *G. gymnothorax*, and both mtDNA and genome data showed that this species is phylogenetically more distant from cave barbs compared to the relationship of cave barbs to each other (Farashi et al. 2014; Hashemzadeh Segherloo et al., 2012, 2017, 2018). Assuming a possible similarity between *12S rRNA* sequences of *G. typhlops* and *G. gymnothorax*, we may not confirm occurrence of *G. typhlops* at localities 13, 17, and 18 due to their direct connection to streams inhabited by *G. gymnothorax*. On the other hand, at locality 10 we detected both Iran cave barb and cave loach eDNA, and this locality is very close to the cave locality, and hence we can confirm the detection of Iran cave barb in this habitat. This ambiguity can be resolved via inclusion of *12S rRNA* sequence of *G. gymnothorax* in the reference databases. Although climatically suitable, at six localities no cave barb eDNA was detected. This negative detection of cave fish should be treated cautiously, since we collected eDNA samples at a distance from the origin of four of these springs along the Tazan and Sirom streams, where increased duration of exposure of eDNA to ultraviolet radiation when flowing on the surface can cause eDNA degradation (Shu et al., 2020; Strickler et al., 2015). Further, cases of negative detection might be related to low concentration of cave fish eDNA in samples, which may be checked by collecting larger water volumes from each locality. A concern regarding negative detection of *G. lorestanensis* in most localities may be differential efficiency of the MiFish primers in amplifying *12S rRNA* for cave barb species due to mismatches between the genomic sequences of these species at primer-binding sites. Although there is no *12S rRNA* sequence data for cave barbs, comparing primer-binding sites between their closely related congener (*G. rufa*) and phylogenetically distant congeners (*G. congoensis*, *G. orientalis*, and *G. salweenica*), no mismatch was identified, which suggests no differential efficiency of MiFish universal primers for amplifying *12S rRNA* fragment in either cave barb species.

4.1 | Distribution

The Iran cave barb was originally recorded from a single locality in the Zagros Mountains, Iran (Bruun & Kaiser, 1944). Later, in 1976, the cave loach was described from the same locality. Mahjoorazad and Coad (2009) reported an undescribed cave barb, which they claimed to be the Iran cave barb or Lorestan cave barb—at the time of their publication, Lorestan cave barb was considered to be conspecific with Iran cave barb—in the Seymareh River in the Karkheh River drainage, 131 km from the original locality in the Dez River drainage. They reported that the specimens had a mental disc, which is lacking in Iran cave barb (Hashemzadeh Segherloo et al., 2012; Sargeran et al., 2008). The Lorestan cave barb also was described from the originally discovered cave fish locality (Mousavi-Sabet & Eagderi, 2016). Vatandoust et al. (2019) reported a new locality for the three described cave fishes in the Dez River drainage, which is 31 km from the original locality in the same river drainage. In the Tang-e-Haft protected area, a specimen of disc-bearing *Garra* sp. was discovered, which resembled a large-size Lorestan cave barb, from a spring located on the west bank of the Sezar River (Dez River drainage) ~4 km from the original locality (Eidi Heidari: Lorestan Province Department of Environment, Lorestan, Iran; unpublished data of the

authors). Additionally, we have found a new locality at a distance of 50–70 km from the original cave barb locality that is inhabited by genetically different fish (unpublished data of I. Hashemzadeh Segherloo). Vatandoust et al. (2019) proposed that the habitat of the cave fishes in the Zagros Mountains might be a network of aquifers extending between 31 and 162 km. As the entire noted 31- to 162-km region falls within the karst formation of the Zagros Mountains and mostly within climatically suitable regions, it is probable that different caves and subterranean habitats have been colonized by members of the genus *Garra*. Our study indicates a more extensive distribution for Iran cave barb that extends to the west from the Dez River drainage, probably to a few localities in the east of the Karkheh River drainage, which is not the case for the Lorestan cave barb and cave loach. Overall, it has been shown using the eDNA approach that subterranean organisms including fishes can have more extensive subterranean distributions than previously recognized (Boyd et al., 2020; Gorički et al., 2017; Vörös et al., 2017; White et al., 2020). For example, using the eDNA (qPCR) approach, White et al. (2020) could detect the Australian blind cave eel *O. candidum* across a wider geographic range than previously known. Overall, we cannot totally confirm the extent of habitat proposed by Vatandoust et al. (2019). There is no doubt for the 31-km southward extent along the Sezar River, where they confirmed the existence of the cave barbs visually and using genetic data, or a nearly 50-km westward distribution, where we detected eDNA only from Iran cave barb that should be confirmed in the follow-up studies. Based on the morphological data presented in Mahjoorazad and Coad (2009), the specimens they found were not Iran cave barb, since their specimens had the mental disc that is absent in Iran cave barb (*G. typhlops*). In addition, it is unlikely that the specimens reported by Mahjoorazad and Coad (2009) were Lorestan cave barb (*G. lorestanensis*), because based on our data, eDNA from Lorestan cave barb was detected only in three springs in the Dez River drainage (the original locality and one other spring to the east). In addition, based on our unpublished data, the disc-bearing cave barbs at 50- to 70-km distance from the original cave barb locality are shown to be genetically different from the cave fish studied here; hence, it would be less probable for fish in a locality 131 km away in the Karkheh River drainage to be Iran or Lorestan cave barb. Based on the hypothesized habitat extent of 31–162 km, Vatandoust et al. (2019) strongly supported down-listing the vulnerable conservation ranking of the Iran and Lorestan cave barbs in IUCN Red List due to their large geographic distribution. However, based on our data, Lorestan cave barb and cave loach are probably limited in distribution to habitats along the Sezar River, and their eDNA was not detected in upper river reaches. Hence, the recommendation to decrease the conservation ranking of the cave barbs should be treated very cautiously, since groundwaters in the basin where these subterranean fishes reside are being heavily depleted by anthropogenic activities (Ashraf et al., 2021). This depletion of the aquifers can increase the vulnerability of the subterranean fishes via habitat loss. Overall, our study cannot support the conservation recommendation by Vatandoust et al. (2019). We propose that performing eDNA metabarcoding across a wider geographic range could be helpful in assessing different hypotheses regarding the distribution, conservation, and taxonomy of the subterranean fishes in the studied region.

4.2 | Habitat use

Hashemzadeh Segherloo et al. (2018), assessing morphological, genetic (nuclear DNA and mitochondrial DNA), and field data, proposed the possibility of sympatric speciation for the Iran and Lorestan cave barbs. They proposed some extent of ecological divergence accompanied with partial niche overlap that made hybridization between the two species possible. According to the field data they collected, Lorestan cave barb (the mental disc-bearing species) appears in the type locality mostly during the fluvial period of the year (late winter-early spring) when flow rate increases. This differential flow-dependent appearance of Lorestan cave barb along with possession of a well-developed mental disc, which is a feature of labeonin fishes inhabiting fast-flowing habitats (Hashemzadeh Segherloo et al., 2017), may imply the tendency of this species to select flowing patches of the subterranean habitats. On the other hand, Iran cave barb (the species without a mental disc) is found in cave barb locality during most of the year. The cave loach was less frequently observed than cave barbs in our field records. The eDNA detected in different springs that showed coexistence of both cave barb species only in the original cave locality supports the partial habitat isolation for the two cave barb species inferred by Hashemzadeh Segherloo et al. (2018). Based on our findings, in the spring located on the west bank of the Sezar River, only Lorestan cave barb and cave loach occur. The frequency of Lorestan cave barb eDNA compared to Iran cave barb at each sampling site decreased from 100% (155 Lorestan cave barb sequence reads versus no Iran cave barb sequence reads) on the Sezar River's bank to 28% (14 Lorestan cave barb sequence reads versus 50 Iran cave barb sequence reads) in the cave locality, but Lorestan cave barb eDNA was not detected in other more westward springs (supporting data V). These results may provide evidence of habitat isolation and a region of hybridization for the cave barbs. As noted, Lorestan cave barb appears in the type locality during the fluvial period, but we performed eDNA sampling in early September 2017, when this species becomes less frequent in the locality. The detection of its eDNA in September when water flow decreases or ceases might be related to eDNA persisting in the water from the fluvial period or real-time occurrence of the fish in lower reaches of the aquifer. Year-round eDNA sampling would be useful to assess seasonal variation and movements of the cave barbs in the subterranean habitat.

4.3 | Species-specific eDNA

Based on our field data and video recorded in the opening of the cave barb locality, the relative frequency of cave loach is less than the frequencies of Iran and Lorestan cave barbs. In contrast to our

direct observation, the frequency of eDNA detected for cave loach in the cave barb locality, its neighboring spring, and in the spring on the west bank of the Sezar River was considerably higher than those for either cave barb species. These results can be explained by alternative hypotheses: (a) cave loach is a benthic fish and not sampled as easily as cave barbs that enter the water column more frequently, (b) cave loach prefers the lower reaches of the subterranean habitat, and (c) cave loach sheds considerably greater amounts of eDNA compared to the cave barbs, perhaps due to the lack of scales (Coad, 2021).

5 | CONCLUSION

Our results suggest that cave fishes were probably present at six new localities in the Dez River and the Karkheh River drainages of western Iran during the sampling period (September 2017). The distribution of cave species also supports a previous hypothesis of partial niche separation between Lorestan cave and Iran cave barbs. Further investigation is needed to better understand spatial and temporal distributions of cave fish communities in the area. As a preliminary study, our results left a few ambiguities related to the effects of the sampling scheme on eDNA detection in the studied system and taxonomic issues (e.g., discrimination of Iran cave barb from the surface-dwelling *G. gymnothorax* with *12S rRNA* sequences) unresolved. We believe that these ambiguities can be resolved via a follow-up study using resampling with replicates and also by producing a comprehensive reference sequence database for species occurring in the study region. Overall, this study demonstrates the utility of eDNA metabarcoding approach as a useful, non-invasive method for providing answers to ecological, taxonomic, and conservation-related questions pertaining to rare fishes in inaccessible subterranean habitats.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

IHS contributed to conceptualization of the study, acquisition, analysis and interpretation of data, writing the manuscript, and getting financial resources, SNT contributed to data acquisition (lab work) and writing the manuscript, EAM contributed to data acquisition (field work), CH contributed to data acquisition (lab work) and analysis, EN contributed to data analysis and interpretation and to writing the manuscript, ML contributed to data interpretation and writing the manuscript, BB contributed to data acquisition (Next Generation Sequencing) and writing the manuscript, MA contributed to data acquisition (field work), NG contributed to data acquisition (field work), EH contributed to writing the manuscript, LB contributed to conceptualization of the study, writing the manuscript, and providing equipment and resources for field and lab work.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available through Dryad: https://doi.org/10.5061/dryad.5dv41ns6x.

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