

## Article

# A New Locality for the Blind Loach, *Eidinemacheilus smithi* (Teleostei: Nemacheilidae) in Iranian Zagros: A Morpho-Molecular Approach

Mohammad Javad Malek-Hosseini<sup>1,2,3,\*</sup>, Yaser Fatemi<sup>4</sup>, Hamid Reza Esmaeili<sup>5</sup>, Tjaša Lokovšek<sup>2</sup> and Matjaž Kuntner<sup>1,2,3</sup> 

<sup>1</sup> Department of Organisms and Ecosystems Research, National Institute of Biology, SI-1000 Ljubljana, Slovenia

<sup>2</sup> Jovan Hadži Institute of Biology, Research Centre of the Slovenian, Academy of Sciences and Arts, SI-1000 Ljubljana, Slovenia

<sup>3</sup> Department of Biology, Biotechnical Faculty, University of Ljubljana, SI-1000 Ljubljana, Slovenia

<sup>4</sup> Department of Marine Biology, Faculty of Marine Sciences and Technology, Hormozgan University, Bandar Abbas 7916193145, Iran

<sup>5</sup> Ichthyology and Molecular Systematics Research Lab., Department of Biology, College of Sciences, Shiraz University, Shiraz 7146713565, Iran

\* Correspondence: malekhosseini1365@gmail.com or javad.malek@zrc-sazu.si

**Abstract:** Four obligate cave fish species have been recorded from Zagros in Iran: *Garra typhlops* (Bruun and Kaiser, 1944), *G. lorestanensis* (Mousavi-Sabet and Eagderi, 2016), *G. tashanensis* (Mousavi-Sabet, Vatandoust, Fatemi and Eagderi, 2016) and *Eidinemacheilus smithi* (Greenwood, 1976). So far, the only known locality of the latter has been the Loven cave. Here, we extend the known range of the Zagros blind loach (*E. smithi*) by reporting a new locality, the Tuveh spring, located 31 km south of Loven. We combine morphological evidence with the calculated K2P genetic divergences of 1.39% between Tuveh and Loven to confirm that these populations are conspecific. Our discovery of the second *E. smithi* population has implications for the conservation of this rare and vulnerable species.

**Keywords:** troglobiont; Zagros blind loach; Irano-Anatolian hotspot; cave; subterranean



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## 1. Introduction

Subterranean environments including caves are among the most extreme ecosystems on earth, as the restricted access to food and the conditions of darkness and humidity make these habitats challenging for living organisms to inhabit. Despite this, many taxa have colonized the subterranean environments, including arthropods and vertebrates [1,2]. The subterranean environments harbor a high diversity of animals and many of them have adapted to life in such habitats. However, regarding the low productivity of hypogean environments, the biodiversity of subterranean environments is much lower than that of the surface. To date, over 22 hotspots of troglomorphic biodiversity, each harboring more than 25 troglobiont/stygobiont species, have been reported globally [2–5]. Unlike numerous troglomorphic invertebrates, e.g., mollusks, sponges, worms, and arthropods [6–8], according to discoveries up to date, only two vertebrate groups, i.e., fishes and salamanders, have colonized subterranean habitats with species that show troglomorphic adaptations [9,10]. With more than 280 known species of troglobiont representatives, the cave fishes are the most species-rich vertebrates in the groundwaters of the world [11]. According to Proudlove [11], the family Nemacheilidae comprises 62 known subterranean species, all but one found in Asia.

The history of Iranian subterranean animal research coincided with railway construction in 1900 [12], with the discovery of the Loven cave in the western Iranian Lorestan Province. At the time, a new cyprinid cave-dwelling species was named *Iranocypris typhlops* (Bruun and Kaiser, 1944), which today is in the genus *Garra* [13–15]. Recently,

Mousavi-Sabet and Eagderi [16] revealed that *Garra* of the Loven cave belong to two distinct species: *G. typhlops* (Bruun and Kaiser, 1944) and *G. lorestanensis* Mousavi-Sabet and Eagderi, 2016. From this locality, Greenwood [17] named another fish species, *Noemacheilus smithi* Greenwood, 1976 (family Nemacheilidae). Hashemzadeh Segherloo et al. [18] transferred the species to *Eidinemacheilus*, a genus of loaches with only two cave-dwelling species: *E. smithi* is endemic to an aquifer in the Karun River drainage in the Zagros Mountains, and *E. proudlovei* Freyhof, Abdullah, Ararat, Ibrahim and Geiger, 2016, is endemic to subterranean waters in the Little Zab River drainage in Iraqi Kurdistan [19].

Mahjoorazad and Coad [20] and Vatandoust et al. [21] reported *G. typhlops* and *G. lorestanensis* from two different localities. The first locality is about 130 km to the west of Loven, and the second one is located 31 km to the south of Loven. These reports may reveal the potential presence of a large freshwater aquifer in the Zagros Mountains. Vatandoust et al. [21] also hypothesized, based on anecdotal and photographic evidence, the presence of *E. smithi* in the Tuveh spring, but this has not been confirmed.

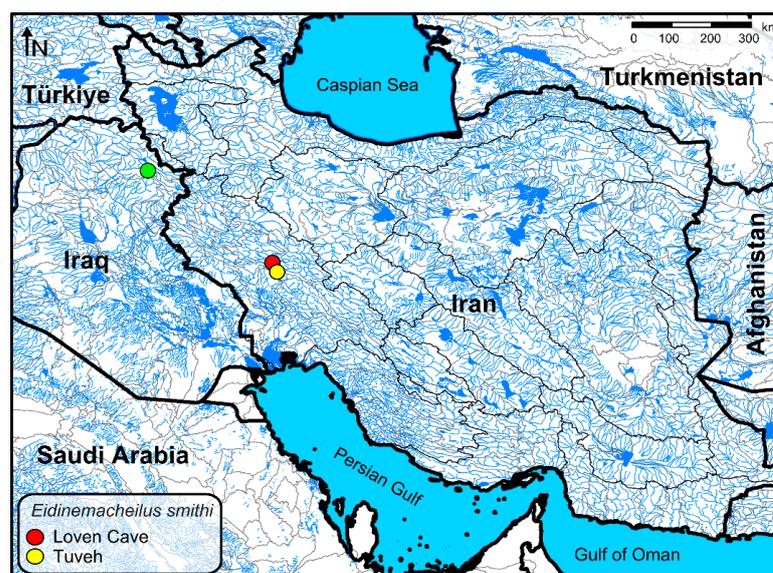
*Garra tashanensis*, Mousavi-Sabet, Vatandoust, Fatemi and Eagderi, 2016, is another subterranean fish species from the Tashan cave in the southeastern part of Iran in Zagros Mountains [22].

Our aims here are: (i) to report a new locality for the Iranian blind loach *Eidinemacheilus smithi*, (ii) to provide morphological characteristics of the collected specimens and a morphological comparison with those from the type locality (Loven cave), and (iii) to use molecular data to test the taxonomic status of these two populations from the Tuveh spring and the Loven cave.

## 2. Material and Methods

### 2.1. Taxon Sampling

Specimens were collected on 30 May 2019, using a hand net in a seasonal spring of Tuveh, close to Tuveh village, Andimeshk, Khuzestan Province ( $32^{\circ}48'47''$  N,  $48^{\circ}43'06''$  E, altitude 495 m a.s.l.) (Figure 1). It is a seasonal spring with a high amount of water flowing during the rainy seasons, usually from November till May–June. The flowing water gets lower from April and the outlet of the spring is terminated completely in summer. The spring is a part of the Dez River drainage. The Tuveh spring is about 31 km to the south of the Loven cave, the type locality of *E. smithi*.



**Figure 1.** Map of Iran showing the two known *Eidinemacheilus smithi* localities. In addition to the previously known locality in red, this paper reports a new locality in yellow. The green circle shows the locality of *Eidinemacheilus proudlovei* in Iraq.

## 2.2. Morphological Study

Measurements were completed using a digital caliper and recorded to 0.1 mm. All of the measurements were made point to point, never by projections. Methods for counts and measurements followed Kottelat and Freyhof [23]. Standard length (SL) was measured from the tip of the snout to the end of the hypural complex. The length of the caudal peduncle was measured from behind the base of the last anal-fin ray to the end of the hypural complex, at mid-height of the caudal-fin base. The last two branched rays articulating on a single pterygiophore in the dorsal and anal fins are noted as “1½”. Caudal peduncle depth was measured at the tip of the posterior-most anal-fin ray.

**Abbreviations.** SL, standard length; HL, head length; ZM-CBSU, Zoological Museum of Shiraz University, Collection of Biology Department, Shiraz, Iran.

## 2.3. Molecular Study

DNA was isolated from the fin tissue of three specimens of *E. smithi* from the Tuveh spring and two specimens of each of the three species of the family Nemacheilidae: *Oxy-noemacheilus euphraticus* (Bănărescu and Nalbant, 1964), *Paraschistura ilamensis* (Vatandoust and Eagderi, 2015), and *Sasanidus kermanshahensis* (Bănărescu and Nalbant, 1966) from Iran. From GenBank, we obtained additional COI sequences of other genera of this family (Table 1). *Lefua nikkonis* (Jordan and Fowler, 1903) was used as the outgroup. GenBank accession numbers are listed in Table 1.

**Table 1.** Taxonomic and gene information (GenBank accession numbers) data for the taxa used in our analyses. Original sequence data are indicated in bold typeface.

No.	Species	Voucher Number and COI Accession Number
1	<b><i>Eidinemacheilus smithi</i> (Tuveh)</b>	<b>F-32: OP310812; F33: OP310813; F34: OP310814</b>
2	<i>Eidinemacheilus smithi</i> 1, 2 (Loven)	KX429660 / KX461958
3	<i>Eidinemacheilus proudlovei</i>	KX774390
4	<i>Nemachilichthys rueppelli</i>	KU928278
5	<i>Oxy-noemacheilus angorae</i>	AP011233
6	<b><i>Oxy-noemacheilus euphraticus</i></b>	<b>F-39: OP310815; F-40: OP310816</b>
7	<i>Oxy-noemacheilus euphraticus</i>	MK546456
8	<i>Oxy-noemacheilus parvinae</i>	KX980092
9	<i>Paracobitis zabgawraensis</i>	MK238776
10	<b><i>Paraschistura ilamensis</i></b>	<b>F-37: OP310817; F-38: OP310818</b>
11	<i>Paraschistura ilamensis</i>	MN258032
12	<i>Paraschistura naumanni</i>	KY808480.
13	<i>Paraschistura nielseni</i>	KY808482
14	<b><i>Sasanidus kermanshahensis</i></b>	<b>F-35: OP310819; F-36: OP310820</b>
15	<i>Sasanidus kermanshahensis</i>	KU928288
16	<i>Schistura beavani</i>	HQ219200
17	<i>Schistura khugae</i>	KJ909375
18	<i>Schistura longa</i>	KM610912
19	<i>Schistura notostigma</i>	NC_031585
20	<i>Schistura poculi</i>	KM610972
21	<i>Schistura savona</i>	KJ542586
22	<i>Triplophysa gundriseri</i>	KX039656
23	<i>Triplophysa orientalis</i>	NC_030505
24	<i>Lefua nikkonis</i>	NC_027662

Molecular procedures were completed at the Evolutionary Zoology Laboratory, Jovan Hadži Institute of Biology ZRC-SAZU, Ljubljana (EZ LAB, Slovenia). The DNA was isolated applying Robotic DNA extraction protocol using Mag MAX™ Express magnetic particle processor Type 700 with DNA Multisample kit (Thermo Fisher Scientific kit) and modified protocols following Videgar et al. [24]. We amplified fragments of the mitochondrial marker: 3' end of cytochrome c oxidase subunit 1 gene (COI) using the primers: VF2\_t1 (5' TGTAACACGACGGCCAGTCAACCAACCACAAAGACATTGGCAC) and FR1d\_t1 (5' CAGGAAACAGCTATGACACCTCAGGGTGTCCGAARAAYCARAA) [25]. PCR reactions were executed in a 35 µL volume using EH2O: 18.8 µL, Buffer: 7.1 µL, dNTPS (2 mM): 3.5 µL, MgCl<sub>2</sub> (25 mM): 3.2 µL, Primer (20 mM): 1 µL forward and 1 µL reverse, polymerase 0.2 µL, and BSA 0.2 µL. The following protocol was applied for PCR: 94 °C for 120 s, 35 cycles of 94 °C for 30 s, 52 °C for 40 s, and 72 °C for 60 s, with a final extension at 72 °C for 10 min. The PCR products were sent to MacroGen Europe (Amsterdam, the Netherlands) for sequencing. We used ChromasPro 2.1.3 (Technelysium, Tewantin, Australia) to assemble the sequences, as well as for editing and proofreading. We used MEGA [26] for alignments and to find the best substitutional model for Bayesian and Maximum Likelihood analyses and as well to estimate K2P Pairwise Distances. For data curation, we used Mesquite version 3.7 [27]. The matrix contained 582 bp. We conducted Bayesian inference using MrBayes v. 3.2.7a [28] to reconstruct the phylogenetic position of the new population of *E. smithi* from the Tuveh spring, its congeners and related genera. The Bayesian inference of phylogeny was conducted for 20 million generations with four MCMC chains and with a sampling frequency of 1000. A relative burn-in was set to 25% and checked for the MCMC chain convergence in Tracer 1.7 [29]. We generated maximum likelihood phylogenetic trees with 1000 bootstrap replicates in IQ-tree 1.6.7 [30]. The analyses were generated under the HKY+G+I model of nucleotide substitution.

#### TAXONOMY

##### Family Nemacheilidae Regan, 1911

##### *Eidinemacheilus smithi* (Greenwood, 1976)

(Figure 2)

**Examined materials.** ZM-CBSU es-101, 2 specimens, 24.6–25.1 mm SL., Iran: Khuzestan Province, Andimeshk, Tuveh village, Tuveh spring, 32°48'47" N, 48°43'06" E.

**Diagnosis.** *Eidinemacheilus smithi* is distinguished from *E. proudlovei*, the only other troglomorphic nemacheilid loach species in the Middle East, and the only other species in *Eidinemacheilus* known so far, by having 7 + 7 branched caudal-fin rays (vs. 8 + 8 or 8 + 7), adipose keel on the caudal peduncle (vs. absent), and a reduced head canal system (fully developed).

**Description.** See Figure 2 for general appearance and Table 2 for morphometric data. Medium sized, moderately stout species with large head. Body deepest at dorsal-fin origin, profile of back straight, or body depth slightly decreasing towards dorsal-fin origin, and slowly decreasing below dorsal-fin base and towards caudal-fin base. No hump at nape. Greatest body width at pectoral-fin base. Head conical, flattened on ventral surface. Caudal peduncle compressed laterally, 1.5–1.6 times longer than deep.



**Figure 2.** *Eidinemacheilus smithi* from Tuveh spring SL: 25 mm.

**Table 2.** Morphometric data of *E. smithi*. ZM-CBSU es-101,  $n = 2$ .

Measured Characters	Min	Max	Mean	SD
Standard length (mm)	24.6	25.1	24.8	
<b>In percent of standard length</b>				
Head length	24	25.4	24.7	1.0
Body depth at dorsal-fin origin	12.6	13.0	12.8	0.2
Body width at dorsal-fin origin	4.6	5.5	5.1	0.6
Pre-dorsal length	46.5	48.5	47.5	1.4
Post-dorsal length	41.8	41.8	41.8	0.01
Pre-pelvic length	51.8	51.9	51.8	0.7
Pre-anal length	73.3	74.3	73.8	0.7
Distance between pectoral and pelvic-fin origins	26.1	27.7	26.9	1.1
Distance between pelvic and anal-fin origins	20.9	23.5	22.2	1.9
Depth of caudal peduncle	9.8	9.9	9.8	0.1
Length of caudal peduncle	14.6	16.0	15.3	0.9
Dorsal-fin length	23.2	24.7	24.0	1.1
Pectoral-fin length	21.8	22.0	21.9	0.1
Pelvic-fin length	12.6	14.4	13.5	1.2
<b>In percent of head length</b>				
Head depth at nape	44.0	45.8	44.9	1.2
Maximum head width	45.6	48.3	46.9	1.9
Inner rostral–barbel length	17.9	18.9	18.4	0.7
Outer rostral–barbel length	39.6	41.5	40.6	1.3
Maxillary–barbel length	25.5	28.2	26.8	1.9

Pectoral fin reaching approximately 79–84% of distance from pectoral-fin origin to pelvic-fin origin. Pelvic axillary lobe absent. Pelvic-fin origin below unbranched or first branched dorsal-fin ray. Pelvic fin not reaching to anus. Anal-fin origin shortly behind anus. Anal-fin origin at vertical of middle between dorsal- and caudal-fin origins. A dorsal adipose crest on caudal peduncle. Margin of dorsal fin straight. Caudal fin moderately emarginated.

Dorsal fin with  $7\frac{1}{2}$  branched rays. Anal fin with  $5\frac{1}{2}$  branched rays. Caudal fin with  $7 + 7$  branched rays. Pectoral fin with 10 and pelvic fin with 5 branched rays. Scales absent. Lateral line complete. Anterior nostril opening on anterior side of a low, pointed, and flap-like tube. Nostrils adjacent to each other, tip of anterior nostril overlaps posterior nostril when folded backwards. No suborbital flap, Groove or slit in fishes examined. Mouth very large, slightly arched. Lips moderately thick, upper lip smooth or with fine ridges, lower lip with deep furrows. A median interruption in lower lip. Barbels short, inner rostral barbell reaching to base or slightly behind of outer rostral barbel; outer rostral barbel reaching to base or slightly behind of maxillary barbel. Maxillary barbel reaching to base of outer rostral barbel when folded to front.

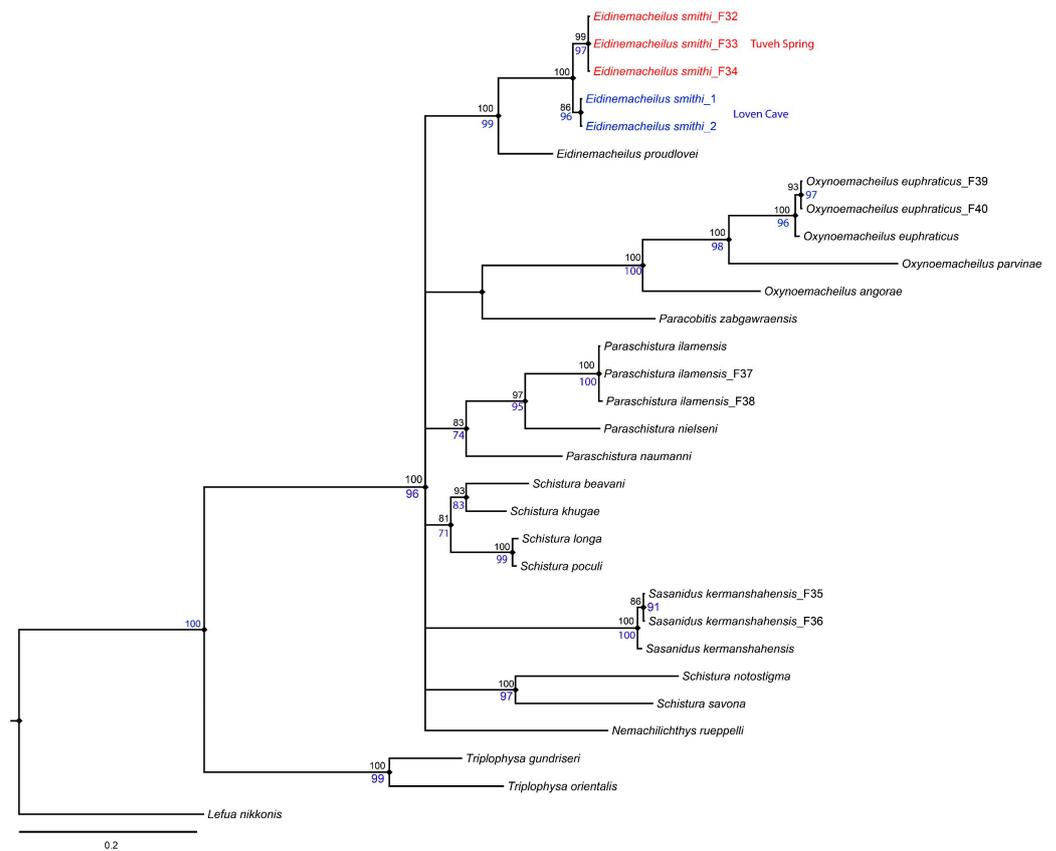
### 3. Molecular Results

The morphological identification was confirmed by DNA barcoding of the specimen collected from the Tuveh spring. Table 3 shows the average estimates of genetic divergence (K2P) based on the COI barcode region among the studied nemacheilid species. *Eidinemacheilus smithi* of both localities (Tuveh and Loven) clustered together, showing a 1.39% K2P distance between these populations. This distance between *E. smithi* of Tuveh and Loven with *E. proudlovei* was 8.06 and 7.67, respectively (Table 3). The topologies from the ML and BI analyses were similar, hence the BI tree including the posterior probability estimates (PP) also plots the bootstrap values from the ML analysis for the clades that both of the analyses have in common (Figure 3). The monophyly of the genus *Eidinemacheilus* with the two described species is confirmed in both of the analyses. *Eidinemacheilus* is placed in a bigger clade containing the genera *Oxynoemacheilus*, *Paraschistura*, *Schistura*, *Sasanidus*, and *Nemachilichthys*. However, the relationships among these genera are unresolved. This clade is sister to the genus *Triplophysa*.

**Distribution.** *Eidinemacheilus smithi* is currently known from its type locality, Loven cave and Tuveh spring, Iran (Figures 2 and 4).

**Table 3.** Estimates of the average COI divergence (K2P distance) between examined Nemacheilidae species.

NO	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	<i>Eidinemacheilus smithi</i> F32-Tuveh																			
2	<i>E. smithi</i> 1- Loven	1.39																		
3	<i>E. proudlovei</i>	8.06	7.67																	
4	<i>Nemachilichthys rueppelli</i>	18.05	18.49	16.52																
5	<i>Oxynoemacheilus angorae</i>	19.30	18.85	19.16	18.76															
6	<i>O. euphraticus</i> _F39	20.07	19.62	18.97	19.82	13.15														
7	<i>O. parvinae</i>	20.49	20.02	18.54	22.56	15.92	12.71													
8	<i>Paracobitis zabgawraensis</i>	17.41	17.19	17.66	15.98	16.70	19.10	20.00												
9	<i>Paraschistura ilamensis</i> _F37	13.75	13.97	15.03	17.77	18.12	20.55	21.96	17.21											
10	<i>P. naumanni</i>	13.97	13.55	13.56	15.78	18.84	17.13	20.80	16.13	11.43										
11	<i>P. nielsenii</i>	13.62	13.84	13.80	18.31	18.62	19.21	22.98	18.87	7.88	12.53									
12	<i>Sasanidus kermanshahensis</i> _F35	16.75	16.53	16.82	18.85	19.61	18.12	21.19	19.68	14.61	16.15	14.95								
13	<i>Schistura beavani</i>	14.11	14.11	12.88	15.95	16.05	15.92	16.58	16.49	13.66	12.01	12.75	14.56							
14	<i>S. khugae</i>	13.08	13.29	12.07	13.57	16.33	15.15	17.35	15.21	12.62	11.04	12.12	15.14	6.48						
15	<i>S. longa</i>	14.13	13.92	11.81	14.05	17.44	16.42	15.98	16.92	13.88	12.07	13.55	14.75	7.66	7.27					
16	<i>S. notostigma</i>	16.69	16.91	16.05	16.51	18.71	19.76	20.42	17.56	18.69	16.14	18.42	17.31	17.19	14.56	16.76				
17	<i>S. poculi</i>	14.53	14.32	12.03	13.61	17.41	16.61	15.95	16.47	14.07	12.46	13.53	15.15	7.84	7.07	0.52	16.52			
18	<i>S. savona</i>	15.83	14.99	16.17	16.33	17.11	18.54	22.19	17.74	15.87	14.60	16.46	18.59	14.38	15.85	16.31	14.71	16.50		
19	<i>Triplophysa gundriseri</i>	20.75	21.92	22.64	21.41	22.18	24.80	26.46	22.52	21.36	19.52	20.58	23.95	17.91	19.03	19.46	22.15	19.65	20.65	
20	<i>T. orientalis</i>	21.96	22.91	23.73	21.62	22.56	25.47	27.57	19.92	18.44	21.40	20.72	22.91	21.77	21.52	22.42	24.31	22.62	20.19	10.21



**Figure 3.** Bayesian and Maximum-Likelihood phylogeny reconstructed based on 582 bp of COI. The values show BI posterior probability (black) and maximum-likelihood (ML; blue) node supports. The topology is from the maximum-likelihood analysis. The values less than 60% are not shown.



**Figure 4.** Tuveh spring in rainy season (A) and the stream path down to the spring in dry season (B).

#### 4. Discussion

A review of available studies suggests the presence of at least eight fish species known to inhabit the subterranean environments of the Middle Eastern countries mainly in the Zagros mountains in the Irano-Anatolian hotspot [11]. These subterranean environments harbor important endemic species. In this study, we used a combined morphological and molecular approach to investigate cave-dwelling fishes of the Zagros Mts, western Iran and reported on a new locality for the Iranian blind loach, *Eidinemacheilus smithi*. The morphological characteristics and K2P distances indicated that the *E. smithi* populations in Tuveh and Loven belong to the same species, genetic and morphological variations notwithstanding. The main problem with our analyses was the unavailability of materials from the Loven cave and data on GenBank other than COI to warrant inclusion of more genetic markers.

*Eidinemacheilus smithi* is distinguished from its congeneric species, *E. proudlovei*, the only other troglomorphic nemacheilid loach species in the Middle East, and the only other species in *Eidinemacheilus* known so far, by having 7 + 7 branched caudal-fin rays (vs. 8 + 8 or 8 + 7), an adipose keel on the caudal peduncle (vs. absent), and a reduced head canal system (fully developed).

Vatandoust et al. [21] reported two cave *Garra* species (*G. typhlops* and *G. lorestanensis*) from the Tuveh spring, known already from the Loven cave which is the type locality for *Garra typhlops* and *G. lorestanensis*. Furthermore, based on the photographed specimens of *E. smithi* they also hypothesized the presence of that species in the Tuveh spring, now confirmed. That these three species inhabit both Tuveh and Loven implies the presence of a large aquifer. We suggest that these cave fishes may be found more widely than is currently known. During floods, for example, a number of specimens or their eggs may be washed out from the Loven cave to other streams, springs, and groundwaters, which facilitates the colonization of new habitats. That said, the 1.39% K2P detected genetic distance between the specimens from Tuveh and Loven does suggest that these populations have been isolated, and so colonization may be a rare event. Vatandoust et al. [21] reported a 0.7% K2P distance in COI between the populations of *Garra lorestanensis* of these two habitats.

Tuveh spring is seasonal and is completely dry during summer. Presumably, the fish specimens die or disperse into rivers. Since we are not aware of any subterranean pools, ponds, or wells around Tuveh spring, these habitats cannot receive immediate protection. Considerable genetic differences between the haplotypes of Tuveh and Loven fishes also preclude any transfers of specimens to the Loven cave. However, the vast area around should be protected to reduce the potential threats for these narrowly endemic species. The Lorestan salamander, *Neurergus kaiseri* Schmidt, 1952, also known from several localities in this area, is considered as vulnerable due to the limited and fragmented range of this species [31,32]. This reinforces the importance of documenting the biodiversity of this area.

Recently, we described a new obligate groundwater species of asellid crustacean from the Ganow spring, about a kilometer east of Tuveh [33], and a new species of trogllobiont gastropod from this aquifer is awaiting description. These findings suggest that increased field efforts will continue to reveal more trogllobiont species inhabiting this big aquifer.

In closing, the Iranian cave fishes from Loven cave are Vulnerable (VU) according to the IUCN list [34–36]. The discovery of new localities for these species keeps us hopeful of preventing the extinction of these vulnerable species. Scientific depredation, droughts and floods, landslides, improper dam constructions, and improper extraction of water from this area are the main threats to the Iranian cave fishes.

**Author Contributions:** Conceptualization, M.K. and M.J.M.-H.; methodology, Y.F., M.J.M.-H. and H.R.E.; software and formal analysis, M.J.M.-H.; all contributed to data compilation, data interpretation, writing, and editing, M.J.M.-H., Y.F., H.R.E., T.L. and M.K. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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