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First blind daddy long-legs spiders from Australia and Réunion (Araneae, Pholcidae)

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Abstract

Daddy long-legs spiders are common inhabitants of tropical and subtropical caves around the globe. Numerous species have evolved troglomorphisms, including the loss of body pigments and eyes. Here we describe the first troglomorphic pholcids from Australia and Réunion. *Belisana coblynau* Huber & Clark, **sp. nov.** was extracted from mining boreholes in the arid West Australian Pilbara region. It represents a genus that is widespread in tropical forests of South and Southeast Asia, reaching the tropical north and east of Australia. *Belisana coblynau* is thus presumably a relict whose epigean ancestor lived in the area before the aridification of Australia starting in the early Cenozoic. *Buitinga ifrit* Huber & Cazanove, **sp. nov.** was collected in Grotte de La Tortue on Réunion, one of the oldest lava tubes on the island (~300,000 years). Congeneric species are known from East Africa, and the genus does not seem to have reached Madagascar. Since Pholcidae do not balloon, the now extinct epigean ancestor of *Buitinga ifrit* probably reached the island by highly accidental means (such as rafts or storms).

Keywords

Lava tube, mining borehole, relict, scrape sample, subterranean, troglomorphism

Introduction

Spiders are in many ways preadapted to a hypogean life: most species rely on mechanical and chemical rather than on visual stimuli, and many species are therefore nocturnal; most spiders endure long periods of starvation, reflecting a low demand of energy; and many species are adapted to near ground microhabitats such as the leaf litter, which share certain characteristics with caves (Foelix 2011; Mammola and Isaia 2017). Among spiders, Pholcidae are one of the most likely groups to be encountered in caves around the globe, in particular in tropical and subtropical regions. A recent review of cave-dwelling pholcid spiders (Huber 2018) found that pholcids have been collected in about 1000 caves, and approximately one fourth of the currently ~1900 described species have been found in caves. However, most of these records are from species that do not appear to be troglomorphic in any obvious way; only 86 species were listed as troglomorphic. Of these, 42 were characterized as "strongly troglomorphic", i.e. without or with reduced eyes (Huber 2018).

An intriguing pattern in subterranean animals is that the large majority of them belong to a relatively small number of major taxa (e.g., Christiansen 2012; Moldovan 2012). This is also the case within Pholcidae: the large majority of troglomorphic Pholcidae are representatives of only two subfamilies: Pholcinae and Modisiminae; the other three subfamilies contain 17% of the known species but only 5% of the troglomorphic species (Huber 2018). This has been thought to explain the apparent absence of troglomorphic Pholcidae in Australia: the large majority of Pholcidae species in Australia are representatives of Arteminae (genera *Pholcitrichocyclus* Ceccolini & Cianferoni, 2022 and *Wugigarra* Huber, 2001), and for unknown reasons, Arteminae around the globe appear extremely unlikely to evolve troglomorphic species. For example, *Physocyclus* Simon, 1893 is a species-rich genus in Mexico, a country with countless caves and numerous troglomorphic Modisiminae (Huber 2018); however, not a single troglomorphic species of *Physocyclus* is known to exist. In Australia, a few representatives of Pholcinae have been described but these were previously thought to be restricted to tropical regions in northern and eastern Australia; they were thought to be relatively recent introductions from SE Asia and New Guinea (Huber 2001). The present finding of a blind *Belisana* Thorell, 1898 (Pholcinae) in the Pilbara supports the idea that Pholcinae rather than Arteminae will be found in available subterranean Australian habitats. At the same time, it refutes the idea that Pholcinae in Australia are restricted to the tropical north and east of the continent.

Another striking observation in troglomorphic pholcid spiders is their over-representation on islands (Huber 2018). Apart from Mexico, which is home to 45% of all known troglomorphic pholcids, most remaining species occur on islands: Jamaica, Canary Islands, Galápagos, Cuba, Madagascar, and Réunion. Since 2018, only one further eyeless pholcid has been described, again from an island (Galápagos; Huber et al. 2022). The eyeless pholcid from Réunion described below was already mentioned in the review of Huber (2018) but it is here formally described.

Materials and methods

Collecting methods

Specimens of *Belisana coblynau* Huber & Clark, sp. nov. were collected by scrape sampling. This method is commonly used to collect troglofauna, particularly when sampling for environmental impact assessments associated with mining (Halse and Pearson 2014). Due to regulations by the Environment Protection Authority (EPA) in Western Australia, new bores that are drilled for mining exploration purposes can only be sampled after six months; as a result, the boreholes were at least six months old at the time of sampling. A weighted net was lowered down uncased holes (diameter 150 mm). The net was lowered to the base of the hole or to the groundwater table (~35 m below ground level) and then drawn slowly back to the surface, "scraping" the net up the wall of the bore, knocking any fauna into the net. This was repeated four times, once on each of the four sides of the bore (i.e., north, south, east, and west). The samples were washed into a 250 ml plastic vial and preserved in 100% ethanol. Samples were stored at 4 °C. *Buitinga ifrit* Huber & Cazanove, sp. nov. was collected manually; specimens were preserved in 70% ethanol and stored at room temperature.

Repositories

The specimens are deposited in the following institutions: Museum d'Histoire Naturelle de La Réunion, Saint Denis (**MHNR**); Western Australian Museum, Perth (**WAM**); and Zoological Research Museum Alexander Koenig, Bonn (**ZFMK**).

Morphology and taxonomy

Taxonomic descriptions follow the style of publications on related Pholcidae taxa (i.e., Huber 2003, 2005; based on Huber 2000). Measurements were done on a dissecting microscope with an ocular grid and are in mm unless noted otherwise. Photos were made with a Nikon Coolpix 995 digital camera (2048 × 1536 pixels) mounted on a Nikon SMZ 18 stereo microscope or a Leitz Dialux 20 compound microscope. CombineZP (https://combinezp.software.informer.com/) was used for stacking photos. Drawings are partly based on photos that were traced on a light table and later improved under a dissecting microscope, or they were directly drawn with a Leitz Dialux 20 compound microscope using a drawing tube. Cleared epigyna were stained with chlorazol black. Abbreviations used in figures only are explained in the figure legends. Abbreviations used in the text: ALS = anterior lateral spinneret(s); a.s.l. = above sea level; L/d = length/diameter.

CO1 barcodes

DNA sequencing of the CO1 gene was conducted on all three *Belisana* specimens collected from the Pilbara, Western Australia. Our effort to extract DNA from *Buitinga* specimens failed. Legs were dissected off the animals for DNA extractions using a Qiagen DNeasy Blood & Tissue kit (https://www.qiagen.com/ie). Elute volumes varied from 40 µl to 200 µl depending on condition and quantity of material. Primer combinations used for PCR amplifications were LCO1490:HCOoutout (Folmer et al. 1994; Schwendinger and Giribet 2005). Dual-direction, Sanger sequencing was undertaken for PCR products by the Australian Genome Research Facility (AGRF). The sequences were edited and aligned in Geneious (Kearse et al. 2012). The three newly sequenced barcodes of *Belisana coblynau* together with the CO1 barcodes of 19 *Belisana* species taken from Eberle et al. (2018) were translated into protein sequences using BioPython (version 1.78) (Cock et al. 2009) with invertebrate mitochondrial genetic code. Next, protein-MSAs were constructed using the mafft-linsi algorithm of MAFFT (version 7.487) (Katoh and Standley 2013), which then assisted the construction of nucleotide level MSAs with pal2nal.pl (Suyama et al. 2006). This helps avoid the introduction of biologically meaningless frameshifts to the alignments (Suyama et al. 2006). The genetic distances between different specimens were calculated based on the Kimura 2-parameter (K2P) model (Kimura 1980) using MEGA11 (Tamura et al. 2021), in which ambiguous positions for each sequence pair were deleted. An initial effort to combine the *Belisana coblynau* sequences with all CO1 Pholcinae sequences from Eberle et al. (2018) and to calculate maximum-likelihood trees was abandoned. The preliminary results from untrimmed and trimmed datasets were highly inconsistent and the suggested affinities not credible (e.g., *Belisana coblynau* nested in the New World endemic genus *Metagonia* Simon, 1893).

Results

Taxonomy

Belisana coblynau **Huber & Clark, sp. nov.**
https://zoobank.org/50A34349-C82F-49FA-8679-77C8CCC86D1B
Figs 1, 2

Type material. Australia – Western Australia • ♂ holotype; ~85 km ESE Pannawonica; 21.8836°S, 117.1211°E; 590 m a.s.l.; 25 Jun. 2019; M.D. Scanlon and H.L. Clark leg.; WAM T158896.

Type locality. Australia, Western Australia, ~85 km ESE Pannawonica, 21.8836°S, 117.1211°E, 590 m a.s.l.

Type specimen. Holotype male, in ethanol. Original labels: “WA: JSE, ca 87 km ESE Pannawonica, 21°53'43.450"S, 117°07'48.63"E (GDA 94) 25. Jun. 2019, Scanlon MD, Clark HL (KRC 0364), Trog scrape, 35 m”, “KRC0364”, “WAM T 158896”, “100% EtOH”.

Other material examined. Australia – Western Australia • 1 ♀, assigned tentatively, see Remark below; same locality as holotype but 3.7 km NW; 21.8583°S, 117.0972°E; 540 m a.s.l.; 25 Apr. 2019; M.D. Scanlon and H.L. Clark leg.;

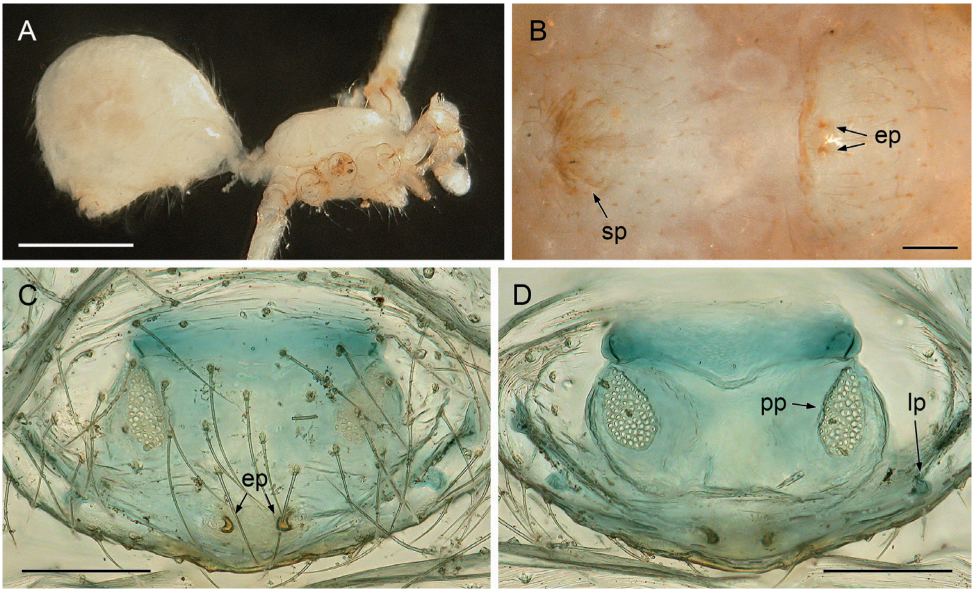

Figure 1. *Belisana coblynau* Huber & Clark, sp. nov., male holotype and female, WAM T158896–7 **A** male habitus, lateral view **B** female abdomen, ventral view **C, D** cleared female genitalia, ventral and dorsal views. Abbreviations: ep, epigynal pockets; lp, lateral pocket; pp, pore plate; sp, spinnerets. Scale bars: 0.5 mm (**A**); 0.1 mm (**B–D**).

"KRC0226", WAM T158897 • 1 juv.; same locality as holotype but 1.6 km SE; 21.8954°S, 117.1302°E; 570 m a.s.l.; 26 Apr. 2019; M.D. Scanlon and H.L. Clark leg.; "KRC0254", WAM T158895.

Remark. The genetic distance (Table 1) between the male holotype and the juvenile specimen was 0.5%, strongly suggesting that the two are conspecific. The distances between these two specimens and the female was much higher, at 8.5 and 8.8%, respectively. This is within a range that in Pholcidae is always problematic due to the overlap of intra- and interspecific genetic (CO1) variation between approximately 8 and 11% (Astrin et al. 2006; Huber et al. in press). In the case of the female, we are thus unsure about its taxonomic status.

Diagnosis. Distinguished from known congeners (and other Pholcinae) by details of procursus (Fig. 2D, E; straight, with simple retrolateral process, bifid prolateral process, and widened membranous tip), genital bulb (Fig. 2A, B; bulbal apophysis short and hooked, embolus straight and pointed at tip), male chelicerae (Fig. 2C; pair of light proximal processes; pair of simple distal frontal apophyses without modified hairs), epigynum (Fig. 1B; protruding, with pair of pockets close together), and female internal genitalia (Figs 1C, D, 2F; pore plates wide apart; distinctive pair of additional internal lateral pockets).

Description. Male (holotype). ***Measurements.*** Total body length ~1.5–1.6 (abdomen detached), carapace width 0.60. Leg 1: 11.5 (2.9 + 0.2 + 3.0 + 4.4 + 1.0); legs 2 and 3 missing; tibia 4: 2.3; tibia 1 L/d: 50.

Table 1. GenBank accession numbers and genetic distances. CO1 accession numbers of the three *Belisana coblynau* specimens and genetic distances (K2P) among them and other *Belisana* species taken from Eberle et al. (2018).

Taxon	OQ525972	OQ525971	OQ525973
OQ525972, *Belisana coblynau* male			
OQ525971, *Belisana coblynau* female	0.088		
OQ525973, *Belisana coblynau* juvenile	0.005	0.085	
S127_Belisana_Bor152_Bor166	0.277	0.261	0.277
S249_Belisana_Mal91_Mal290	0.285	0.283	0.287
S242_Belisana_leuser_Mal304	0.233	0.241	0.233
S247_Belisana_Mal76_Mal278	0.274	0.299	0.276
S498_Belisana_minneriya_SL123	0.238	0.245	0.236
S248_Belisana_Mal77_Mal279	0.277	0.274	0.275
S103_Belisana_Tai4_Tai67	0.233	0.264	0.231
S230_Belisana_australis_Phi280	0.244	0.264	0.244
S125_Belisana_Bor121_Bor198	0.227	0.241	0.225
S244_Belisana_Mal30_Mal241	0.255	0.269	0.259
S333_Belisana_bohorok_Ind127	0.250	0.250	0.248
S336_Belisana_nahtanoj_Ind181	0.209	0.212	0.208
S338_Belisana_tambligan_Ind213	0.237	0.234	0.234
S339_Buitinga_buhoma_Uga124	0.254	0.269	0.251
S340_Buitinga_ruhiza_Uga122	0.252	0.262	0.247
S341_Buitinga_ruwenzori_Uga156	0.255	0.269	0.249
S334_Belisana_Ind15_Ind140	0.274	0.256	0.274
S386_Belisana_sabah_Bor214	0.235	0.250	0.233
S417_Belisana_ranong_Mal361	0.210	0.215	0.210

Color (in ethanol). Entire specimen pale ochre to whitish (Fig. 1A).

Body. Habitus as in Fig. 1A. Ocular area not raised, without any remnants of eye pigment and lenses. Without thoracic groove. Clypeus unmodified. Sternum wider than long (0.40/0.30), unmodified. Abdomen globular, conical at spinnerets.

Chelicerae. As in Fig. 2C, with pair of light proximal processes, pair of simple distal frontal apophyses without modified hairs (distance between tips: 50 µm), without stridulatory files.

Palps. As in Fig. 2A, B; coxa unmodified, trochanter with low rounded retrolateral ventral protrusion, femur cylindrical, without process; tibia with only one (retrolateral distal) trichobothrium; procursus (Fig. 2D, E) straight, with simple retrolateral process, bifid prolateral process, and widened membranous tip; genital bulb with short hooked bulbal apophysis and straight embolus pointed at tip.

Legs. Apparently without spines and curved hairs (most hairs on available legs missing); leg trichobothria not seen; tarsus 1 with ~10 pseudosegments, barely visible in dissecting microscope.

Female. In general similar to male, but larger (in particular the abdomen); total body length 1.8; carapace width 0.62; most legs detached (tibia 4: 2.6). Epigynum (Fig. 1B) main anterior plate semicircular, with distinct pair of pockets (distance between pockets: 30 µm), in lateral view protruding. Internal genitalia (Figs 1C, D, 2F)

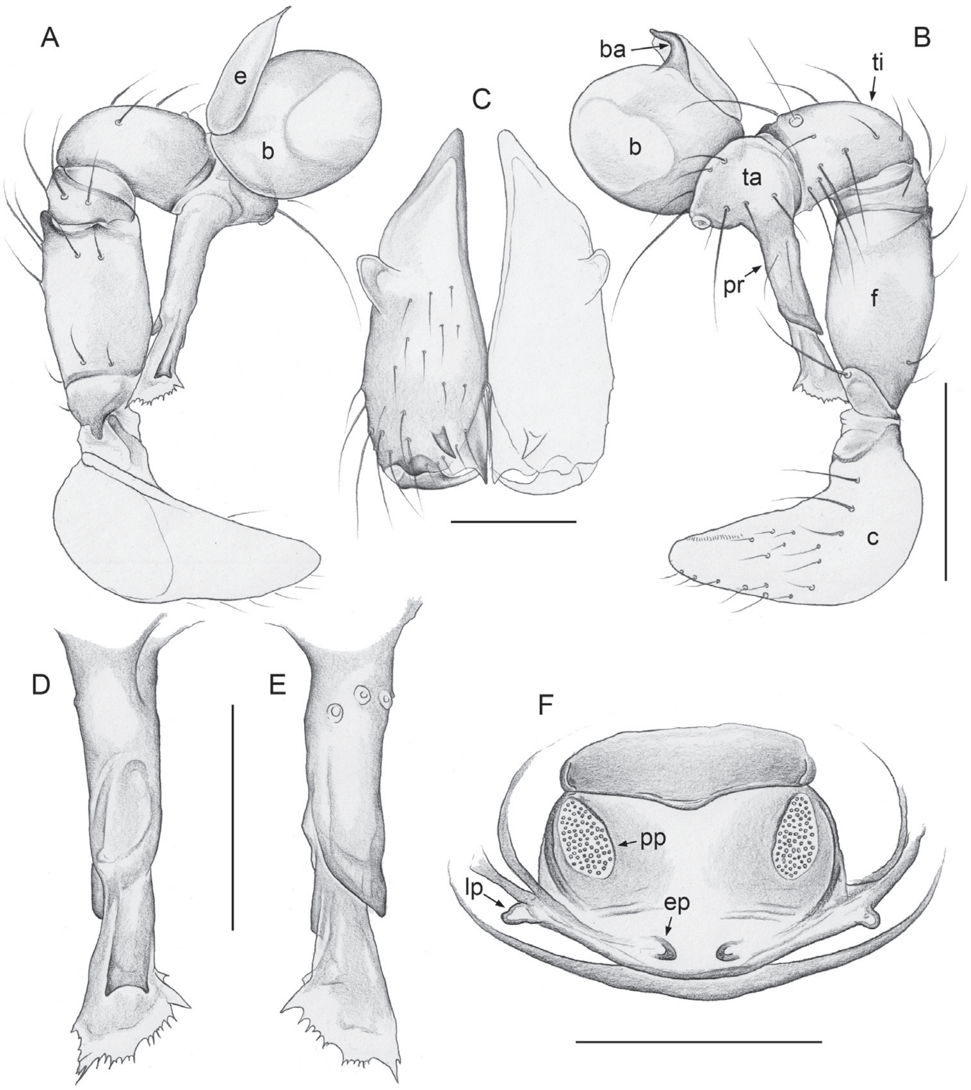

Figure 2. *Belisana coblynau* Huber & Clark, sp. nov., male holotype and female, WAM T158896–7 **A, B** left male palp, prolateral and retrolateral views **C** male chelicerae, frontal view **D, E** left procursus, prolateral and retrolateral views **F** cleared female genitalia, dorsal view. Abbreviations: b, genital bulb; ba, bulbal apophysis; c, coxa; e, embolus; ep, epigynal pocket; f, femur; lp, lateral pocket; pp, pore plate; pr, procursus; ta, tarsus; ti, tibia. Scale bars: 0.2 mm (**A, B, F**); 0.1 mm (**C–E**).

with pair of pore plates wide apart, distinctive pair of additional internal lateral pockets. Each ALS with 3–4 small cylindrical spigots in addition to large widened spigot and long pointed spigot (checked in cleared abdomen).

Etymology. The species epithet refers to the mythical gnome-like creatures that are said to haunt mines and quarries (of Wales and beyond); noun in apposition.

Distribution. Known from three neighboring (within 5 km) localities in Western Australia.

Natural history. The spiders were extracted from mining bores in the Hamersley Range using scrape sampling (Fig. 5A–C). This collecting method precludes gaining detailed natural history data. The specimens were collected between ground level and approximately 35 m below ground level. The temperature in these holes at 15 m below ground level was constant at 32–33 °C. For a general characterization of the Pilbara troglofauna, see Discussion.

Buitinga ifrit Huber & Cazanove, sp. nov.

https://zoobank.org/094155C6-26E5-47A3-8D88-AE84E1D7873D
Figs 3, 4

Buitinga? sp. nov. "Reun1": Huber 2018: 4.

Type material. Réunion – Saint-Paul • ♂ holotype; Grotte (Caverne) de la Tortue; 21.0743°S, 55.2491°E; ~220 m a.s.l.; 9 Mar. 1997; J.-C. Ledoux leg.; ZFMK (Ar 24187).

Type locality. Réunion, Saint-Paul, Grotte (Caverne) de la Tortue, 21.0743°S, 55.2491°E, ca 220 m a.s.l.

Type specimen. Holotype male, in ethanol. Original label: "St. Paul, grotte de la Tortue, Pholcidae (sur toile) 9/3/1997".

Other material examined. Réunion – Saint-Paul • 1 ♂ 6 ♀ (+ 1 juv.) paratypes; same data as for holotype; MHNR • 1 ♀ paratype (+ 1 juv.); same locality as for holotype, Salle du Muséum; 13 Jul. 1996; C. Guillermet leg.; between stones from scree which obstruct the bottom of the gallery, "2022.E.6.2"; ZFMK (Ar 24188) • 1 ♀ paratype; same locality as for holotype; Salle du Muséum; 24 Feb. 2007; S. Gasnier leg.; on the ground, "CT/070124.004" "2011.E.65.1"; MHNR.

Diagnosis. Distinguished from known congeners (and other Pholcinae) by strong bifid process on male palpal trochanter (Fig. 4B), by details of procursus (Fig. 4E, F; short main branch curved towards dorsal and with deeply bifid tip, large and mostly membranous side branch originating from ventral side of main branch, with complex tip), genital bulb (Fig. 4A; bulbal apophysis slender and weakly curved, with small process at basis, embolus cylindrical), male chelicerae (Fig. 4C, D; pair of light proximal processes; pair of distal frontal apophyses with four modified hairs each), epigynum (Fig. 3B; barely protruding, without pockets, distinctive median posterior process only visible in cleared specimens), and female internal genitalia (Figs 3C, D, 4G; oval pore plates; pair of dark lateral structures).

Description. Male (holotype). ***Measurements.*** Total body length 2.0, carapace width 0.70. Leg 1: 18.5 (4.6 + 0.3 + 5.1 + 7.3 + 1.2); tibia 2: 3.1; tibia 3: 1.9; tibia 4: 2.8; tibia 1 L/d: 85.

Color (in ethanol). Entire specimen pale ochre to whitish (Fig. 3A).

Body. Habitus as in Fig. 3A. Ocular area slightly raised, without any remnants of eye pigment and lenses. Without thoracic groove. Clypeus unmodified. Sternum wider than long (0.50/0.40), unmodified. Abdomen oval, conical at spinnerets.

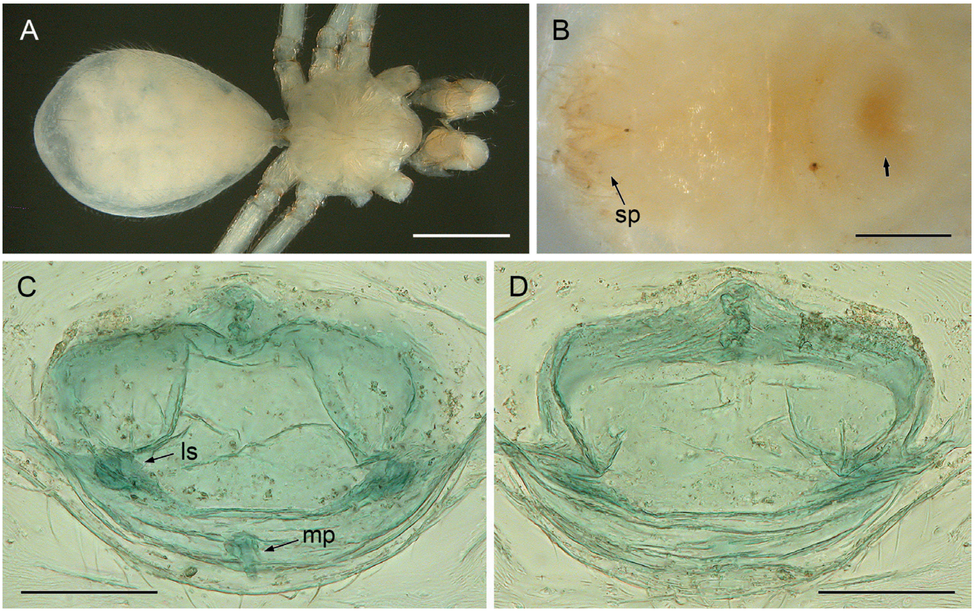

Figure 3. *Buitinga ifrit* Huber & Cazanove, sp. nov., male holotype and female paratype, ZFMK (Ar 24187–88) **A** male habitus, dorsal view **B** female abdomen, ventral view (arrow: darker median area) **C, D** cleared female genitalia, ventral and dorsal views. Abbreviations: ls, dark lateral structure; mp, median posterior process; sp, spinnerets. Scale bars: 0.5 mm (**A**); 0.2 mm (**B**); 0.1 mm (**C, D**).

Chelicerae. As in Fig. 4C, with pair of light proximal processes and pair of distal frontal apophyses with four modified hairs each (Fig. 4D); without stridulatory files.

Palps. As in Fig. 4A, B; coxa unmodified, trochanter with strong bifid process, femur short, slightly widening distally, without process; tibia with two trichobothria; procursus (Fig. 4E, F) complex, with short main branch curved towards dorsal and with deeply bifid tip, large and mostly membranous side branch originating from ventral side of main branch, with complex tip; genital bulb with slender and weakly curved bulbal apophysis with small process at basis, and cylindrical embolus with transparent fringes at tip.

Legs. Apparently without spines and curved hairs (many hairs missing); few short vertical hairs; leg trichobothria and pseudosegments not seen.

Variation (male). Second male apparently indistinguishable; legs 1 missing.

Female. In general similar to male; total body length 1.8–2.0; carapace width 0.68–0.72; tibia 1 in seven females: 3.7–4.7 (mean 4.1). Epigynum (Fig. 3B) main anterior plate slightly protruding, barely visible in ventral view, with darker median area, without pockets, with distinctive median posterior process (Fig. 4H, I) only visible in cleared specimens. Internal genitalia (Figs 3C, D, 4G) with barely visible oval pore plates and pair of dark lateral structures. Each ALS apparently with only two spigots (large widened spigot and long pointed spigot; checked in cleared abdomen).

Etymology. The species epithet refers to a demon in Islamic mythology that is often associated with the underworld; noun in apposition.

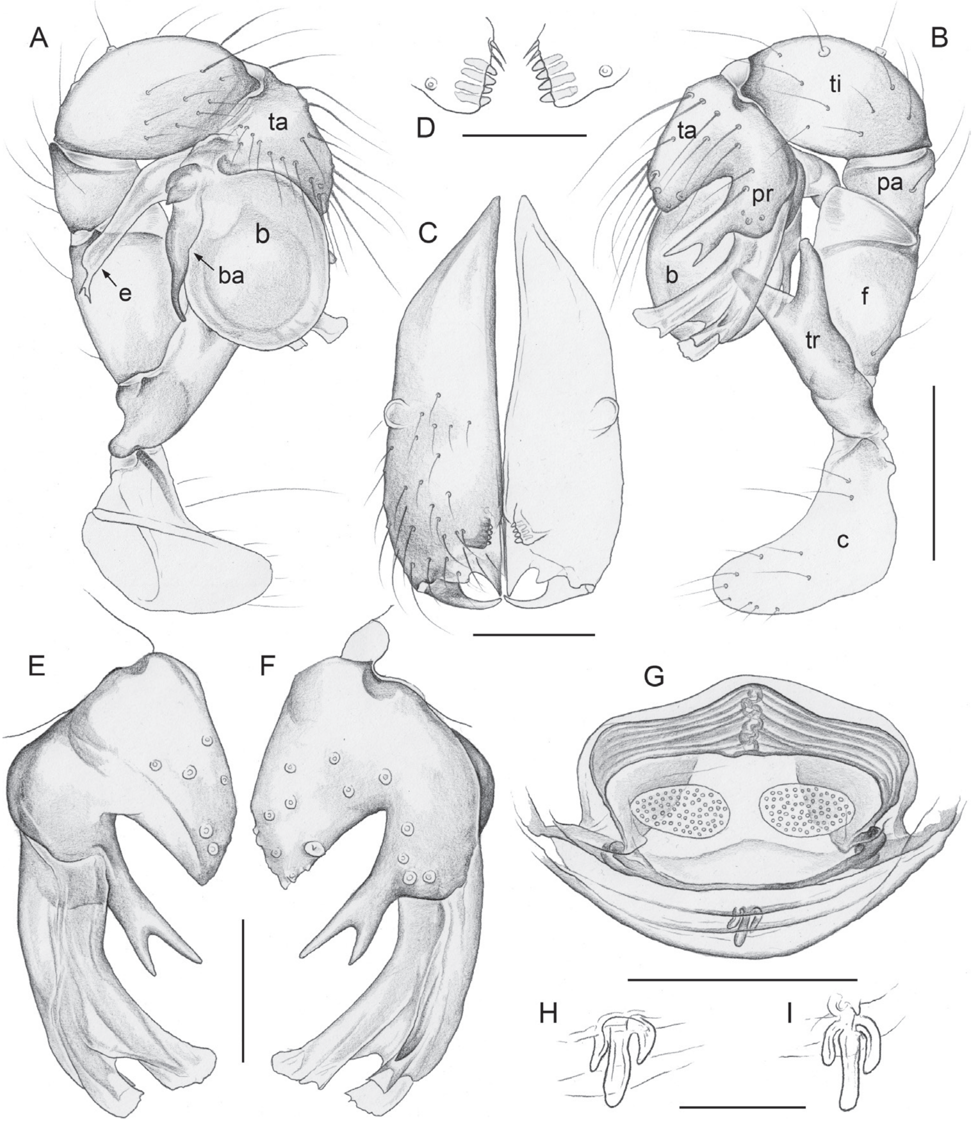

Figure 4. *Buitinga ifrit* Huber & Cazanove, sp. nov., male holotype and female paratype, ZFMK (Ar 24187–88) **A, B** left male palp, prolateral and retrolateral views **C, D** male chelicerae, frontal view, and frontal male cheliceral apophyses in more detail **E, F** left tarsus and procursus, prolateral and retrolateral views **G–I** cleared female genitalia, dorsal view, and posterior median structure in two cleared specimens in more detail. Abbreviations: b, genital bulb; ba, bulbal apophysis; c, coxa; e, embolus; f, femur; pa, patella; pr, procursus; ta, tarsus; ti, tibia; tr, trochanter. Scale bars: 0.2 mm (**A, B, G**); 0.1 mm (**C, E, F**); 0.05 mm (**D, H, I**).

Distribution. Known from type locality only.

Natural history. The spiders were collected deep within the Grotte de la Tortue lava tube (Fig. 5D, E). Some of the specimens were collected in the Salle du Muséum,

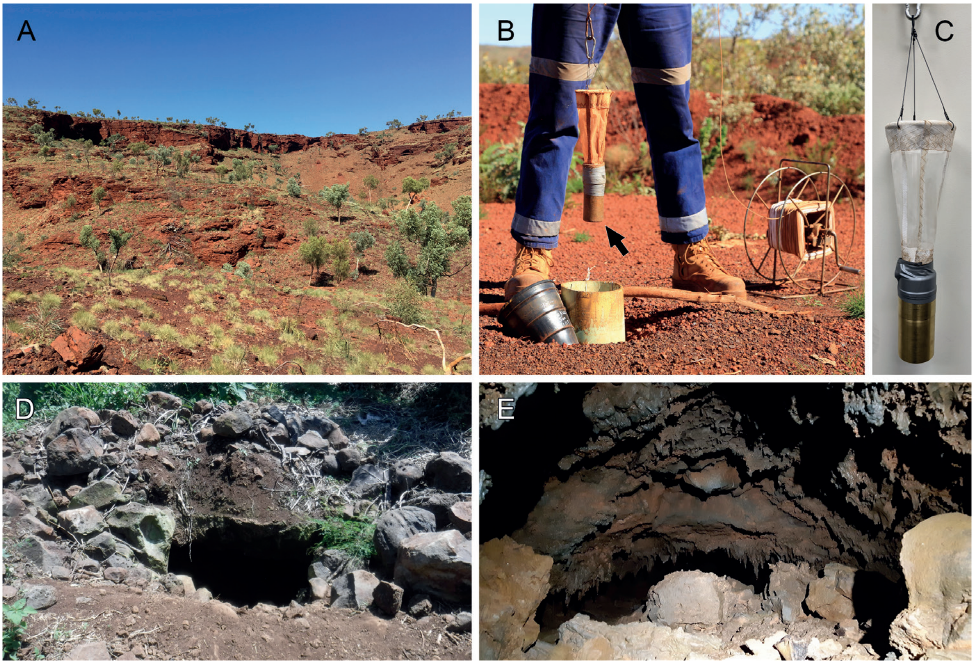

Figure 5. Epigean and hypogean habitats and collecting method **A** Hamersley Range in Western Australia; the area of the sampled boreholes **B** weighted net (arrow) above a borehole **C** weighted net used for scrape sampling **D** main entrance of the Grotte de La Tortue (photo T. Percheron, 2015) **E** gallery of the lost goat in Caverne de La Tortue (photo T. Percheron, 2022).

a section that is about 150 m from the cave entrance (see topographic map in Hoch et al. 2003). They were found among rocks on the ground. For the other specimens, cave section and microhabitat are unknown. For a general characterization of the caves and cave fauna of Réunion, see Discussion.

Discussion

Generic placements and their implications

The taxonomic assignments of the new species to the genera *Belisana* Thorell, 1898 and *Buitinga* Huber, 2003 are not immediately obvious and need some justification, especially since this affects the biogeographic interpretation. Both species are clearly representatives of Pholcinae, based on the proximal lateral processes on the male chelicerae. Within Pholcinae, they are very likely representatives of what has been called Pholcinae 'group 1' or 'group 2' (Huber et al. 2018), i.e. Pholcinae without a distinct sclerite connecting the male genital bulb to the tarsus. These two groups together currently count 16 genera.

For the new Australian species, numerous genera of Pholcinae 'group 1' and 'group 2' can be ruled out easily: several have only two spigots on each ALS (*Aetana* Huber, 2005; *Hantu* Huber, 2016; *Khorata* Huber, 2005; *Paramicromerys* Millot, 1946; *Savarna* Huber, 2005; *Spermophorides* Wunderlich, 1992; *Wanniyala* Huber & Benjamin, 2005); in some genera, the genital bulb has only one process, the embolus (*Anansus* Huber, 2007; *Giloloa* Huber, 2019; *Metagonia* Simon, 1893; *Nyikoa* Huber, 2007; *Tangguoa* Yao & Li, 2021); females of *Spermophora* Hentz, 1841 have one or two copulatory pockets behind the epigynum; in *Buitinga*, males have frontal cheliceral apophyses with modified hairs and females have an epigynal scape; and in *Zatavua* Huber, 2003 males, the lateral cheliceral apophyses point backwards and there is a retrolateral notch on the palpal tarsus. This leaves only *Belisana*, which is, together with *Spermophora*, the only member of Pholcinae 'group 1' and 'group 2' known to occur in Australia. However, no known species of *Belisana* has a procursus that has a specific similarity with the procursus of *Belisana coblynau* Huber & Clark. The large genetic distances to all other sequenced *Belisana* species (and to other Pholcinae) support the isolated position of this species. Clearly, more molecular data are needed to support or refute our generic assignment. However, the main conclusion is unaffected by the exact generic placement: *Belisana coblynau* is a relict species. Except for *Belisana coblynau*, all extant Australian Pholcinae are restricted to the tropical north and east of the continent (Huber 2001, maps 15–19). Certain Pholcinae taxa must have been present in Australia before the climate became drier and more seasonal between 25 and 10 Ma (Martin 1994), or at least before severe aridity set in during the Pliocene (5–2 Ma) (Bowler 1982; Crisp et al. 2004).

Belisana coblynau is the fourth troglomorphic representative of the genus; the three other species are from Thailand, Laos, and Vietnam (Huber 2018). Compared to a sample of 146 described and undescribed congeners (B.A. Huber, unpublished data), *B. coblynau* is close to the mean values regarding carapace width, tibia 1 length, and metatarsus 1 length. With respect to body size and leg length, the species is thus apparently not troglomorphic.

For the new species from Réunion, most of the possible genera can also be ruled out easily. The following is a selection of diagnostic characters: in numerous genera, frontal male cheliceral apophyses are either absent or they have no modified hairs (*Aetana*, *Anansus*, *Belisana*, *Giloloa*, *Hantu*, *Khorata*, *Nyikoa*, *Savarna*, *Tangguoa*); in some genera, females consistently have epigynal or abdominal copulatory pockets (*Anansus*, *Belisana*, *Nyikoa*, *Paramicromerys*, *Spermophorides*, *Spermophora*, *Zatavua*); in some genera, the ALS have numerous (5–6) spigots rather than only two (*Anansus*, *Nyikoa*, *Zatavua*); in some genera, the genital bulb has only one process, the embolus (*Anansus*, *Giloloa*, *Metagonia*, *Nyikoa*, *Tangguoa*); all known representatives of *Wanniyala* have a modified male clypeus and long and widely spaced frontal cheliceral apophyses. This leaves only *Buitinga* (incl. its sister group, a clade of misplaced East African "*Spermophora*"; Huber et al. 2018), an East African genus in which males of most species have cheliceral apophyses that are very similar to those of the newly described species and in which females are characterized by a scape (even though the shape of the scape in *Buitinga ifrit* Huber & Cazanove is unique). Surprisingly, however, no known *Buitinga* in East Africa has a procursus that has a specific similarity with the unique procursus of *Buitinga ifrit*. If our generic assignment is correct, then the ancestor of *Buitinga ifrit* must have reached Réunion from East Africa within the last few million years. This is remarkable for two reasons: first, Pholcidae do not easily reach Far Islands (Huber and Meng 2023); second, *Buitinga* is not known to occur on Madagascar. *Buitinga ifrit* is the only known troglomorphic representative in the genus.

Australian troglofauna without caves

According to White (1988), a cave can be defined as "a natural opening in the Earth, large enough to admit a human being, and which some human beings choose to call a cave". The Pilbara (and Yilgarn) troglofauna is unique in that it inhabits a landscape devoid of caves that would fit White's definition (Halse 2018a, b). Instead, these animals occupy faults, fractures, and voids that form micro-caverns (<5 mm width) and meso-caverns (5–500 mm), which are widespread throughout the landscape (Howarth 1983). These subterranean spaces occur in the host rock of the vadose zone, defined as the space between ~2 m below the ground surface and the water table (Halse and Pearson 2014). The main mechanism resulting in these subterranean spaces is weathering. It is caused largely by water flow, temperature change and animal movement. The related mineralization process whereby minerals are leached from some areas of host rock to enrich other areas can also create voids (Morris 1983). Due to the extreme age of the Pilbara (ca. 3,660–2,800 Ma; Wellman 2000), there are extensive areas within rock formations where subterranean spaces appropriate for subterranean fauna have developed (Johnson 2009).

The Hamersley Range is one of the most prominent features in the Pilbara landscape, and consists largely of exposed banded iron formation (Fig. 5A), a geology known to have been extensively weathered and to contain suitable habitat for troglofauna (Johnson and Wright 2001). These environments have a stable temperature and constant humidity (Moldovan et al. 2018), in contrast to surface conditions where high temperatures and dry conditions are commonplace. It has been suggested that the troglofauna of this region has been forced into the subterranean world by the aridification of the Australian continent (Humphreys 2000; Harvey et al. 2008). Early in the Cenozoic, water was plentiful throughout Australia, but over the course of ~60 Ma, the majority of inland surface water has been lost, with accelerating aridification in the last 33 Ma (Mabbutt 1977; Crisp et al. 2004; Owen et al. 2017). The absence of light and the minimal availability of organic matter (Moldovan et al. 2018) has led to the usual adaptations seen in cave animals: deficient pigmentation, reduced or absent eyes, vermiform bodies, elongate sensory structures, loss of wings, increased lifespan, K-selected breeding strategies, and decreased metabolic rates (Gibert and Deharveng 2002).

Troglofaunal spiders of the Pilbara are estimated to have ranges of between 1 and ~1,400 km^2 (Halse and Pearson 2014) with a median linear range of 2.2 km (Halse 2018b). For comparison, troglofaunal isopods are understood to have a median range of 1.8 km while dipterans and hemipterans have greater median ranges of 68 and 159 km, respectively (Halse 2018b). The vast majority of troglofauna species have small ranges (< 10,000 km^2) and meet Harvey's (2002) criteria of Short-Range Endemism (SRE).

The Pilbara is one of the richest regions of the world for troglofauna, with over 1,500 species estimated as of 2018 (Halse 2018b). Approximately 7% of collected species from the Pilbara are spiders (Halse 2018b). At least 91 species of spiders are represented within the troglofauna of the Pilbara including the newly discovered pholcid (Bennelongia Environmental Consultants, unpublished data). Of these, the goblin spiders of the family Oonopidae are the most represented in both number of genera (6), number of species (50) and number of specimens (139). All of the described species of troglofaunal spiders of the Pilbara are goblin spiders, with eight species of *Prethopalpus* described by Baehr et al. (2012). Additionally, the goblin spider *Opopaea ectognophus* was described by Harvey and Edward (2007) along with two other species from the Cape Range and Kimberley regions of Australia, respectively. An additional goblin spider, *Pelicinus saaristoi* has been described by Ott and Harvey (2008) from Barrow Island, a subterranean fauna hotspot located off the Pilbara coast (Clark et al. 2021; Eberhard and Howarth 2021). Apart from these few studies, troglofaunal spiders of the Pilbara remain understudied and underrepresented in the scientific literature. Unpublished data suggest the presence of a rich spider fauna, including Gnaphosidae, Linyphiidae, Micropholcommatidae, Symphytognathidae, Tetrablemmidae, Theridiidae, and Trochanteriidae (Bennelongia Environmental Consultants, unpublished data).

Caves and cave fauna in Réunion

Réunion is a volcanic island and its underground environment is largely limited to lava tubes dating back to approximately 300,000 years (Sendra et al. 2017). Several large lava tubes at low altitudes are documented in Audra (1997). The Grotte de La Tortue volcanic tube is located on the northwestern side of the island, at the western base of the now inactive Piton des Neiges volcano. The cave is a lava tunnel system developed within vesicular basaltic lava. The ground hosting this cavity comes from the flows of phase II of the Piton des Neiges (estimated age between 2,100,000 and 430,000 years ago; Brial 1996). It is one of the oldest known caves on Réunion Island. With a maximum length of 322 meters, the cavern system is complex, with many branches and narrow sections (see topographic map in Hoch et al. 2003). The cumulative length of the galleries explored to date is approximately 690 m (Brial 1996). The main entrance is steep and accessible only by ladder or abseil; a second entrance is accessible only by crawling in the Gallery of the Toad. The first cavern forms a large chamber but quickly grades into narrow, shallow passages. At the main entrance, fallen blocks of basalt cover the ground but starting at approximately 2 m from the entrance, these are covered by very fine sediment (50–150 cm thick). This sediment is continuous through the cave system. The cave atmosphere is extremely humid (>97% according to Hoch et al. 2003). The temperature is 25–26 °C (Guillermet 1996; Cazanove and Mahé 2007). Some of the cave floor deposits show evidence of high-energy fluvial activity (ripple marks on shallow compact sediments). Brial (1996) speculated that a lake may form during heavy rains. According to Hume (2005), the cave was a natural pitfall trap for a number of endemic species because several animal bones were found, such as the famous Bourbon Giant Turtle, *Cylindraspis indica* (Schneider, 1783) and flightless birds (Mourer-Chauviré et al. 1999).

Despite a few studies (Rochat et al. 2003; Cazanove and Mahé 2007), the subterranean fauna on Réunion Island is still poorly known. Previously, only one troglobitic spider species has been known from the island: *Trogloctenus briali* Ledoux, 2004 (Ctenidae) (Rochat et al. 2003; Ledoux 2004), which is known from a single female specimen collected in Grotte de La Tortue. Other troglobites documented from Réunion include a planthopper (Hoch et al. 2003), Staphylinidae and Carabidae beetles (Jarrige 1957; Deuve 2007; Poussereau et al. 2011; Lecoq 2012) and a diplurid (Sendra et al. 2017). Unpublished records for the Grotte de La Tortue include unidentified representatives of Pseudoscorpiones, Acari, Myriapoda, Coleoptera, Blattoptera (Cazanove and Mahe 2007; G. Cazanove, unpubl. data), as well as two introduced spider species: *Eidmanella pallida* (Emerton, 1914) (Nesticidae) and *Loxosceles rufescens* (Dufour, 1820) (Sicariidae) (J.C. Ledoux, pers. comm. 2013).

Conclusions

The newly described *Belisana coblynau* Huber & Clark from the arid Pilbara in northwestern Australia is the first troglomorphic pholcid spider reported from the continent. It represents the subfamily Pholcinae, which was previously thought to be restricted to the tropical north and east of the continent. The new species suggests that Pholcinae were instead widely distributed in Australia before the continent's aridification in the last tens of millions of years.

The newly described *Buitinga ifrit* Huber & Cazanove from a lava tube on Réunion Island has its closest known relatives in eastern Africa. The genus *Buitinga* is not known to occur on Madagascar. Together with the age of Réunion (~3 Ma) and the Grotte de La Tortue lava tube (~300,000 years), this suggests that the epigean ancestor of *Buitinga ifrit* has reached Réunion relatively recently and by highly accidental means (such as rafts or storms), and that it adapted relatively quickly to the subterranean environment.

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RESEARCH ARTICLE

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The surprising discovery of two new subterranean Leptodirini of the genus *Spelaeobates* Müller, 1901 (Coleoptera, Leiodidae, Cholevinae) from Croatia after more than a century

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Abstract

Two new subterranean leiodid taxa of the genus *Spelaeobates* Müller, 1901 from three pits in northern Dalmatia (Croatia), *S. coriniensis* **sp. nov.** and *S. coriniensis nonveilleri* **ssp. nov.**, are described. The morphological traits of the new taxa are enumerated and illustrated. These two taxa are placed in the subgenus *Spelaeobates* Müller, 1901. The relationships of these two taxa and their close relatives are clarified. Data on the sexual dimorphism of the two new taxa and on the intrasubspecific variability of *S. coriniensis nonveilleri* **ssp. nov.** are presented. We also redescribed *S. novaki*, the type species of both the genus *Spelaeobates* and the subgenus *Spelaeobates* and the closest relative of the newly described species. A key for identification of the taxa of the genus *Spelaeobates* is included. The new taxa are endemic to the Dinaric Alps of Croatia. *Spelaeobates* (*Pretneriella*) *kraussi* Müller, 1903 and *S.* (*P.*) *pharensis langhofferi* Müller, 1931 were found for the first time outside their type locality.

Keywords
Balkan Peninsula, Dinarides, hypogean, leiodids, new species, new subspecies

Introduction

The genus *Spelaeobates* Müller, 1901 (Coleoptera, Leiodidae, Cholevinae, Leptodirini) includes the following six troglobitic species from Dalmatia (Croatia): *Spelaeobates bachofeni* Breit, 1913 (from an unnamed cave, near the town of Vis, island of Vis); *S. czernyi* Breit, 1913 (from another unnamed cave, near the town of Vis, island of Vis; the Špilja od Vore Cave and the Špilja na Bardorovici Cave, village of Kostirna, near the town of Komiža, island of Vis); *S. kraussi* Müller, 1903 (from the Dobra Jama Pit, Vidova Gora peak, village of Nerežišća, island of Brač); *S. peneckei* Müller, 1903 (from the Činjadra Špilja Cave, village of Škrip, island of Brač; Ješkalovica Pit and Studentski Ratac Pit, village of Selca, island of Brač; Kopačina Cave, village of Nerežišća, near the town of Supetar, island of Brač; and Ježulje Cave, village of Donji Humac, near the town of Supetar, island of Brač); *S. pharensis* Müller, 1901, with two subspecies: *S. pharensis pharensis* Müller, 1901 (from the Špilja pod Kapelu Cave, Smokovnik hill, island of Hvar; Jama u Pečarovu Stanu Pit, Propod Pit and "Neue *Spelaeobates* Höhle bei Lesina" Cave, near the town of Hvar, island of Hvar; and Jama u pod Kuse Pit, village of Brusje, island of Hvar) and *S. pharensis langhofferi* Müller, 1931 (from the Kruščica Cave, near the town of Stari Grad, island of Hvar); and *S. novaki* Müller, 1901 (from the Strašna Peć Cave, village of Savar, island of Dugi Otok; and Jezero Cave, village of Mali Iž, island of Iž) (Fig. 1) (Müller 1901, 1903, 1931; Breit 1913; Jeannel 1924; Pretner 1973; Guéorguiev 1990; Perreau 2000, 2015; Hlaváč et al. 2017). Perreau (2000, 2015) reported the presence of *S. novaki* only from the island of Iž, but it actually inhabits the nearby island of Dugi Otok as well (Pretner 1973; Guéorguiev 1990; Hlaváč et al. 2017). This genus is chiefly distributed on islands in northern (Dugi Otok and Iž) and central Dalmatia (Brač, Hvar, and Vis) in Croatia (Fig. 1) (Pretner 1973; Guéorguiev 1990; Perreau 2000, 2015; Hlaváč et al. 2017). Jalžić (1982) reported one finding of this genus from the mainland of northern Dalmatia (Croatia) (from the Golubnjača Cave, village of Kaštel Žegarski, near the town of Obrovac), indicating that the genus is not distributed only on the Dalmatian islands. However, it wasn't specified to which subgenus the taxon found in the Golubnjača Cave belongs (Jalžić 1982). The genus is highly specialised and is considered to be very old (Guéorguiev 1990). It comprises small-sized subterranean taxa with a narrow head, a narrow pronotum, convex pear-shaped physogastric elytra, and a very small, narrow, elongate, simple and unarmed endophallus (Jeannel 1924). The genus *Spelaeobates* is divided into two subgenera: *Spelaeobates* Müller, 1901 and *Pretneriella* V. Guéorguiev, 1976. The subgenera differ on the degree of punctuation on the pronotum, width of the first protarsomere in males, shape of the median lobe apex, and number of the parameral setae (Guéorguiev 1976). The nominotypic subgenus includes only the species *S. novaki*, while the subgenus *Pretneriella* comprises all remaining taxa of the genus *Spelaeobates*. The former subgenus is distributed only on two islands (Dugi Otok and

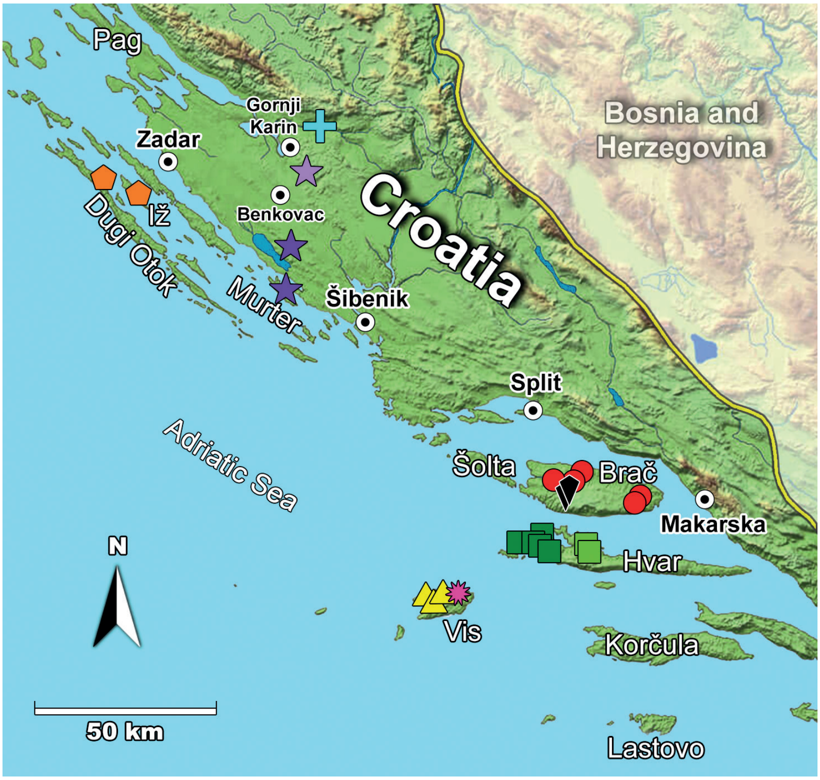

Figure 1. A map of part of Dalmatia (Croatia) showing localities where *Spelaeobates* taxa were registered **pink sun** *S.* (*P.*) *bachofeni* **yellow triangles** *S.* (*P.*) *czernyi* **black kites** *S.* (*P.*) *kraussi* **red circles** *S.* (*P.*) *peneckei* **lime squares** *S.* (*P.*) *pharensis langhofferi* **green squares** *S.* (*P.*) *pharensis pharensis* **light purple star** *S.* (*S.*) *coriniensis coriniensis* ssp. nov. **purple stars** *S.* (*S.*) *coriniensis nonveilleri* ssp. nov. **orange pentagons** *S.* (*S.*) *novaki* **light blue cross** *S.* sp.

Iž) belonging to northern Dalmatia, while the latter is recorded on three islands (Brač, Hvar, and Vis) in central Dalmatia. Generally, the genus *Spelaeobates* inhabits both the islands and mainland of northern and central Dalmatia (Croatia) and its taxa are subterranean (Perreau 2000, 2015). It is interesting to note that all known species of this genus were described in a short time interval at the beginning of the 20th century (from 1901 to 1913). The taxon of this genus which was described most recently (92 years ago) is the subspecies *S. pharensis langhofferi* (Perreau 2000, 2015).

The fauna of caves and pits of central and southern Dalmatia has been very well explored (Pretner 1973; Jalžić and Pretner 1977), while the biospeleological research of northern Dalmatia has been carried out on a very small scale, so the findings of numerous new taxa of subterranean leioids to science can be expected in this area.

Three field trips in northern Dalmatia (Croatia) conducted by the Špiljar Speleological Society (Split, Croatia) in 2019 at three subterranean sites resulted in the discovery of a number of adult individuals of two new leptodirine leiodid taxa (one species and one subspecies) belonging to the nominotypic subgenus of the genus *Spelaeobates*, whose descriptions and diagnoses are presented in the current study. In 1997, the same team collected several adult specimens of the species *S.* (*S.*) *novaki* at its type locality (Strašna Peć Cave, village of Savar, island of Dugi Otok), which is redescribed in this paper.

Materials and methods

The adults of leptodirine leiodid beetles were collected manually in three pits and one cave in the vicinity of the town of Benkovac and the city of Šibenik, as well as on the island of Dugi Otok (northern Dalmatia, Croatia). These beetle individuals were studied in a laboratory of the Institute of Zoology, University of Belgrade - Faculty of Biology, Belgrade, Serbia. Their genitalia were extracted and conserved on microscope slides in a medium consisting of Canada balsam and toluene. Beetles were then glued on paper mounting cards and examined as dry specimens. We measured a total of seven individuals (three males and four females) of *S.* (*S.*) *coriniensis* sp. nov., nine individuals (five males and four females) of *S.* (*S.*) *coriniensis nonveilleri* ssp. nov., and four individuals (one male and three females) of *S.* (*S.*) *novaki* (values are given as averages and ranges in the Results). Taxonomically important morphological traits were studied for comparison using a Stemi 2000 binocular stereomicroscope (Carl Zeiss, Jena, Germany), a SMZ 18 binocular stereomicroscope (Nikon, Tokyo, Japan) with a DS-Fi1c digital camera (Nikon, Tokyo, Japan) appended, as well as an Axioskop 40 light microscope (Carl Zeiss, Jena, Germany). A Nikon DS-L3 control unit was used for scale bar adjustment and precise measurements. An Intralux 5100 cold light source (Volpi, Schlieren, Switzerland) was used for the additional illumination of beetles under binocular stereomicroscopes. A Tescan Mira 3 XMU field emission scanning electron microscope (FESEM) (Tescan, Brno, Czech Republic) at the University of Belgrade - Faculty of Technology and Metallurgy was used for observing the detailed morphology of the new taxa. Prior to analysis, beetle samples were coated with gold for 45 s using a Polaron SC502 Sputter Coater (Fisons, VG Microtech, East Sussex, UK). The high-vacuum mode of the FESEM at an acceleration voltage of 10 kV was used. The index of electron beam intensity was 8.00, the electron beam current was 364 µA, while the pressure in the column was around 1.3e–3 Pa.

The systematics used follow Jeannel (1924), Perreau (2000, 2015), and Hlaváč et al. (2017).

Abbreviations of measurements

A3L/A2L	ratio of length of antennomere III to length of antennomere II;
A3L/A4L	ratio of length of antennomere III to length of antennomere IV;
A6L/A3L	ratio of length of antennomere VI to length of antennomere III;
A8L/A3L	ratio of length of antennomere VIII to length of antennomere III;
A11L/A8L	ratio of length of antennomere XI to length of antennomere VIII;
A11W/A10W	ratio of width of antennomere XI to width of antennomere X;

AL	total antennal length (including the scape);
EL/EW	ratio of elytral length (as the linear distance measured along the suture from the elytral base to the apex) to maximum elytral width;
EW/PW	ratio of maximum elytral width to maximum pronotal width;
HL/HW	ratio of head length to maximum head width;
HW/PW	ratio of maximum head width to maximum pronotal width;
M	mean value;
M1L/M1W	ratio of length to width of maxillary palpomere I;
M2L/M1L	ratio of length of maxillary palpomere II to length of maxillary palpomere I;
M3L/M2L	ratio of length of maxillary palpomere III to length of maxillary palpomere II;
PB/AM	ratio of pronotal base length to anterior pronotal margin length;
PL/HL	ratio of pronotal length to head length;
PL/PW	ratio of pronotal length to maximum pronotal width;
P1W/P2W	ratio of width of protarsomere I to width of protarsomere II;
R	range of total measurements performed;
TL	total body length (measured from the anterior margin of the clypeus to the elytral apex).

Collections

IZFB	collection of the Institute of Zoology, University of Belgrade - Faculty of Biology, Belgrade, Serbia;
SSM	collection of the Split Science Museum, Split, Croatia.

Other abbreviations

HT	holotype;
PT	paratype;
TR	leg. Tonći Rađa.

Other examined taxa

Spelaeobates (*Pretneriella*) *kraussi* Müller, 1903: one male, one female, Croatia, central Dalmatia, island of Brač, village of Nerežišća, Vidova Gora peak, Vičja Jama Pit, 16.IV.2022, TR (IZFB).

Spelaeobates (*Pretneriella*) *pharensis langhofferi* Müller, 1931: two females, Croatia, central Dalmatia, island of Hvar, town of Jelsa, village of Pitve, Jama na Boroviku Pit, 9.XI.2013, TR (IZFB).

Spelaeobates (*Spelaeobates*) *novaki* Müller, 1901: one male, four females, Croatia, northern Dalmatia, island of Dugi Otok, village of Savar, Strašna Peć Cave, 1.VII.1997, TR (IZFB).

Results

Order Coleoptera Linnaeus, 1758
Family Leiodidae Fleming, 1821
Subfamily Cholevinae Kirby, 1837
Tribe Leptodirini Lacordaire, 1854
Subtribe Spelaeobatina V. Guéorguiev, 1974
Genus *Spelaeobates* Müller, 1901
Subgenus *Spelaeobates* Müller, 1901

***Spelaeobates* (*Spelaeobates*) *coriniensis* Ćurčić, Vesović, Vrbica & Rađa, sp. nov.**

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Figs 2, 3, 6

Type material. ***Holotype*:** male (SSM) labeled as follows: "CROATIA, NORTHERN DALMATIA: town of Benkovac, settlement of Gornji Karin, village of Popovići, Jamurka (Rnjakuša II) Pit, 216 m a.s.l., 44°04'44.1"N, 15°41'00.3"E, 8.II.2019, TR" (white label, printed) / "Holotypus *Spelaeobates* (*Spelaeobates*) *coriniensis* sp. nov. Ćurčić, Vesović, Vrbica & Rađa det. 2022" (red label, printed).

Paratypes (three males and five females). The same data as for HT (IZFB). All paratypes are labeled with white, printed locality labels and with red printed labels "Paratypus *Spelaeobates* (*Spelaeobates*) *coriniensis* sp. nov. Ćurčić, Vesović, Vrbica & Rađa det. 2022" (Fig. 2).

Etymology. *Spelaeobates* (*Spelaeobates*) *coriniensis* sp. nov. is named after Corinium, a Roman town in the area of today's Gornji Karin, a settlement close to its type locality.

Diagnosis. The new species is most closely related to another species of *Spelaeobates* s. str., *S. novaki*, by the rimmed lateral pronotal margins, the presence of a dilated first protarsomere in males, a low, unangled mesoventral carina, the presence of its apically attenuated median lobe of the aedeagus, and by the presence of four parameral setae (Figs 2, 3, 7, 8) (Müller 1901; Jeannel 1924; Guéorguiev 1976).

Spelaeobates (*S.*) *coriniensis* sp. nov. is easily distinguished from *S.* (*S.*) *novaki* in terms of TL (R 2.37–2.50 mm vs. R 2.50–2.80 mm), length of antennae when stretched backwards (reaching end of elytra vs. not reaching end of elytra), length of first two antennomeres (antennomere II longer than antennomere I vs. antennomeres I and II approximately equal in length), A6L/A3L (M 0.93, R 0.88–1.00 vs. M 0.79, R 0.76–0.82), A8L/A3L (M 0.85, R 0.75–0.93 vs. M 0.65, R 0.59–0.71), A11L/A8L (M 1.94, R 1.77–2.08 vs. M 2.27, R 2.09–2.40), maximum width of head (between first quarter and third vs. between first third and middle), punctuation on pronotum (fine, punctures separated vs. strong, punctures merged), EL/EW in males (R 1.56–1.71 vs. R 1.53), maximum width of elytra (before middle vs. around at middle), EW/PW (R 2.15–2.34 vs. R 2.37–2.50), width of first protarsomere in males (less broadened vs. more broadened), and shape of median lobe of aedeagus in dorsal view (more narrowed distally, pointed apically vs. gradually narrowed distally, almost sub-parallel, rounded apically) and in lateral view (less curved basally, almost straight apically vs. more curved basally, slightly bent downward apically) (Table 1, Figs 2, 3, 7, 8) (Müller 1901; Jeannel 1924; Guéorguiev 1976).

Description. Small-sized leptodirine. TL M 2.42 mm (2.43 mm in males, 2.41 mm in females), R 2.37–2.50 mm (2.37–2.50 mm in both males and females).

***Habitus*:** Body shape leptodiroid (Fig. 2A, B), colour yellowish.

***Integument*:** Lustrous, microsculptured both dorsally and ventrally (Fig. 2C, D, F–L). Sparsely distributed deep punctures present on head, while densely distributed, fine and separated on both pronotum and elytra (Fig. 2C, D, F, G, K, L). Entire body dorsally covered with yellow pubescence of short length (erect on head, while recumbent on both pronotum and elytra) (Fig. 2A, B).

***Head*:** About one and a half times as long as wide (HL/HW M 1.49, R 1.37–1.65), more elongate in males (HL/HW M 1.52 in males, M 1.46 in females), with no eyes, occipital carina in the shape of a curved concave line (Fig. 2A, C). Head widest between first quarter and third. Frons roundly impressed between antennal insertions. Labrum transverse, with a few long setae. First maxillary palpomere of similar length and width, shorter than second maxillary palpomere. Maxillary palpomeres II and III of similar length (M3L/M2L M 1.02, R 0.85–1.09). Penultimate maxillary palpomere widened apically. Ultimate maxillary palpomere short, slender, gradually narrowing apically. Antennae inserted in basal quarter of head, thin, narrow proximally (except for first two antennomeres, which are thickened), slightly widened distally, longer in males, AL M 1.83 mm, R 1.71–1.97 mm (1.88–1.97 mm in males, 1.71–1.82 mm in females),

Table 1. Linear measurements and morphometric ratios in *Spelaeobates* (*Spelaeobates*) *coriniensis coriniensis* ssp. nov., *S.* (*S.*) *coriniensis nonveilleri* ssp. nov., and *S.* (*S.*) *novaki*. Values outside parentheses are mean values, while those inside parentheses are ranges.

Species/subspecies	***S.* (*S.*) *coriniensis coriniensis* ssp. nov.**	***S.* (*S.*) *coriniensis nonveilleri* ssp. nov.**	***S.* (*S.*) *novaki***
Character			
TL*	2.42 (2.37–2.50)	2.46 (2.34–2.59)	2.63 (2.57–2.70)
HL/HW	1.49 (1.37–1.65)	1.57 (1.53–1.61)	1.47 (1.38–1.53)
HW/PW	0.89 (0.87–0.92)	0.91 (0.87–0.97)	0.93 (0.88–0.98)
AL*	1.83 (1.71–1.97)	1.86 (1.77–1.94)	1.81 (1.75–1.93)
A3L/A2L	1.26 (1.15–1.33)	1.34 (1.23–1.45)	1.37 (1.23–1.42)
A3L/A4L	1.09 (1.00–1.15)	1.21 (1.07–1.31)	1.24 (1.21–1.31)
A6L/A3L	0.93 (0.88–1.00)	0.86 (0.81–0.88)	0.79 (0.76–0.82)
A8L/A3L	0.85 (0.75–0.93)	0.75 (0.65–0.81)	0.65 (0.59–0.71)
A11L/A8L	1.94 (1.77–2.08)	2.03 (1.77–2.25)	2.27 (2.09–2.40)
A11W/A10W	0.78 (0.67–0.83)	0.84 (0.67–1.00)	0.75 (0.67–0.83)
M3L/M2L	1.02 (0.85–1.09)	0.97 (0.85–1.09)	1.09 (1.00–1.18)
PL/PW	1.24 (1.21–1.28)	1.30 (1.24–1.38)	1.29 (1.26–1.32)
PL/HL	0.95 (0.89–0.98)	0.91 (0.89–0.96)	0.96 (0.90–0.98)
PB/AM	0.86 (0.81–0.93)	0.89 (0.83–0.96)	0.86 (0.81–0.90)
EW/PW	2.25 (2.15–2.34)	2.28 (2.18–2.36)	2.44 (2.37–2.50)
EL/EW in males	1.64 (1.56–1.71)	1.64 (1.55–1.76)	1.53 (1.53)
P1W/P2W in males	1.75 (1.50–2.00)	1.20 (1.00–1.50)	1.67 (1.67)

* – values in mm.

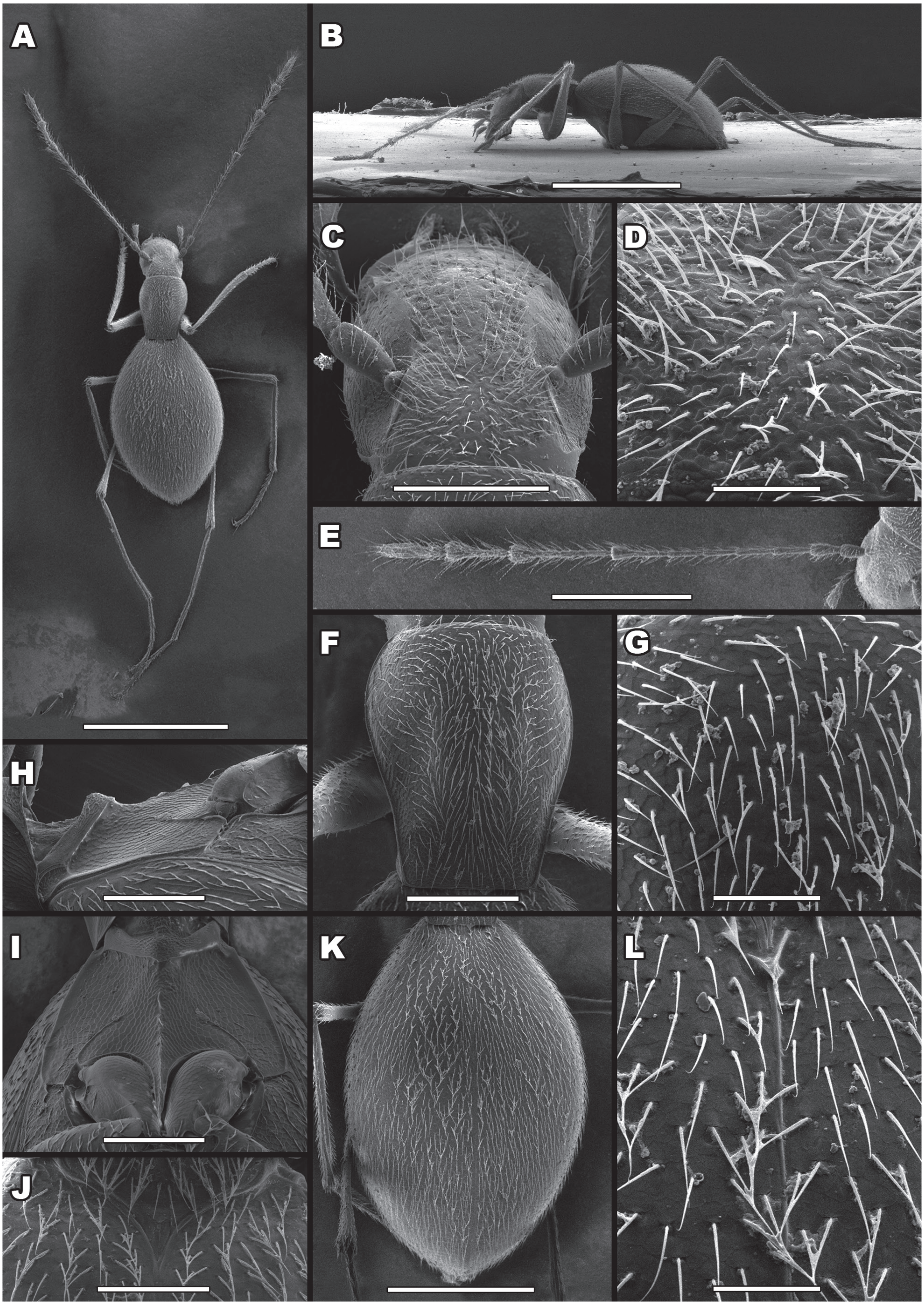

Figure 2. SEM images of the morphological structures of PT male (**A–G, J–L**) and PT female (**H, I**) of *Spelaeobates* (*Spelaeobates*) *coriniensis* sp. nov. from the Jamurka (Rnjakuša II) Pit, village of Popovići, settlement of Gornji Karin, town of Benkovac, northern Dalmatia, Croatia **A** habitus, dorsal view **B** habitus, lateral view **C** head, dorsal view **D** microsculpture of head, dorsal view **E** right antenna, dorsal view **F** pronotum, dorsal view **G** microsculpture of pronotum, dorsal view **H** mesoventral carina, lateral view **I** mesoventrite, ventral view **J** scutellum, dorsal view **K** elytra, dorsal view **L** microsculpture of elytra, dorsal view. Scale bars: 1.0 mm (**A, B**); 0.5 mm (**E, K**); 0.2 mm (**C, F, H, I**); 0.1 mm (**J**); 0.05 mm (**D, G, L**).

reaching end of elytra in males (Fig. 2A, B, E). Antennomeres I and II short and wide, second of which slightly longer and narrower. Following four antennomeres thinner and slightly longer than antennomere II. Antennomere III longer than adjacent antennomeres (A3L/A2L M 1.26, R 1.15–1.33; A3L/A4L M 1.09, R 1.00–1.15). Antennomeres VII, IX, and X quite expanded distally. Antennomere VIII relatively short and narrow, shorter and narrower than anatennomeres VII, IX, X, and XI. Ultimate antennomere slender, widened sub-distally, then narrowing apically, narrower than penultimate one (A11W/A10W M 0.78, R 0.67–0.83). Antennomere I shortest, while antennomeres IX and XI longest. Other ratios of length of certain antennomeres: A6L/A3L M 0.93, R 0.88–1.00; A8L/A3L M 0.85, R 0.75–0.93; A11L/A8L M 1.94, R 1.77–2.08.

***Prothorax*:** Pronotum bell-shaped, elongate, longer than wide (PL/PW M 1.24, R 1.21–1.28; M 1.26, R 1.24–1.28 in males; M 1.23, R 1.21–1.27 in females), widest around anterior third, broader (HW/PW M 0.89, R 0.87–0.92) and shorter than head (PL/HL M 0.95, R 0.89–0.98) (Fig. 2A, F). Lateral margins rounded anteriorly, after which they constrict towards posterior end, slightly concave posteriorly. Pronotal base straight, somewhat shorter than elytral base. PB/AM M 0.86, R 0.81–0.93. Anterior margin barely convex medially, almost straight. Lateral margins and pronotal base rimmed. Fore pronotal angles weakly expressed, rounded, obtuse. Hind pronotal angles well-expressed, obtuse, not protruding backwards. Pronotal disc moderately convex (Fig. 2B).

***Mesothorax*:** Mesoventral carina very low, barely noticeable, with a few setae (Fig. 2H). No tooth, anterior and posterior margins observed. Mesoventrite with a long process between mesocoxae, which is gradually narrowing apically (Fig. 2I). Scutellum large, sub-triangular (Fig. 2K, J).

***Metathorax*:** Metaventrite without carina.

***Elytra*:** Wide, ovoid, almost of same width in males and females (EL/EW M 1.64, R 1.56–1.71 in males; M 1.64, R 1.60–1.69 in females), markedly wider than pronotum (EW/PW M 2.25, R 2.15–2.34) (Fig. 2A, K). Maximum width a little before middle. Lateral margins arcuate. Marginal furrows not visible from above. Shoulders barely visible, obtuse, covered by hind pronotal angles. Elytral disc markedly convex, steeply declining both basally and apically in lateral view (Fig. 2B). Parasutural stria absent. Elytral apex slightly attenuated, rounded. Pygidium covered by elytra.

***Legs*:** Elongate and slender (Fig. 2A, B). Femora widened basally, constricted in distal half. Tibiae thin, gently curved, gradually widening distally. Each protibia with a very fine comb over entire apical third of outer margin. Fore tarsi four-segmented in both sexes, only first protarsomere in males dilated (P1W/P2W M 1.75, R 1.50–2.00). Tarsal claws thin, elongate, curved, pointed apically.

***Male genitalia*:** Aedeagus elongate, slender, small, well chitinised (Fig. 3A, B). Median lobe in dorsal view straight, gradually narrowing distally, with a sharp apex, markedly longer than parameres (Fig. 3A). Median lobe in lateral view quite flattened, curved basally, almost straight proximally, narrowing apically (Fig. 3B). Basal bulb small, narrow, sub-parallel, slightly widened distally and bilobed in dorsal view (Fig. 3A), while elongate and widened basally in lateral view (Fig. 3B). Tegmen wide from above (Fig. 3A), in the shape of a ring around basal bulb (Fig. 3B). Parameres elongate, slender, arcuate, sub-apically curved exteriorly, each with a moderately widened rounded

Figure 3. Bright-field images of the male genitalia of HT of *Spelaeobates* (*Spelaeobates*) *coriniensis* sp. nov. from the Jamurka (Rnjakuša II) Pit, village of Popovići, settlement of Gornji Karin, town of Benkovac, northern Dalmatia, Croatia **A** aedeagus, dorsal view **B** aedeagus, lateral view. Scale bars: 0.1 mm.

apex in dorsal view (Fig. 3A), while straight, sub-parallel in lateral view (Fig. 3B). Each paramera bearing four apical close-set setae, two of which longer, while two shorter (Fig. 3A). No copulatory piece observed within inner sac (Fig. 3A, B).

***Female genitalia*:** Spermatheca small, chitinised, curved, markedly constricted medially, spherical both basally and apically (Fig. 6A). Gonostyli short, straight, moderately widened, gradually narrowing distally, pointed apically (Fig. 6B). Each gonostylus carrying one long apical seta.

***Male abdominal sternite IX (urite)*:** Small, narrowing apically, sub-triangular.

***Female abdominal ventrite VIII*:** Small, transverse, with no anterior process, hairy, especially posteriorly.

Sexual dimorphism. Some degree of sexual dimorphism was noted in this new species. Namely, it was found that: (i) antennae are longer in the males than in the females; (ii) antennomeres VIII–X are more elongate in the males than in the females; (iii) head is more elongate in the males than in the females; (iv) pronotum is slightly more elongate in the males than in the females; (v) first protarsomere is wider in the males than in the females.

Type locality. Jamurka (Rnjakuša II) Pit, village of Popovići, close to the settlement of Gornji Karin and the town of Benkovac, northern Dalmatia, Croatia.

Geographic distribution. The new species inhabits a few pits in the vicinity of the town of Benkovac and the city of Šibenik in northern Dalmatia (Croatia). Its type locality, the Jamurka (Rnjakuša II) Pit, represents the northernmost location of a *Spelaeobates* species. At the same time, this is the first official finding of a species of the genus *Spelaeobates* on the mainland, which confirms that this genus is distributed both on the islands and on the mainland of Dalmatia (Jalžić 1982). It is possible that the new species also inhabits the surrounding subterranean habitats in northern Dalmatia.

Bionomy and habitat. Individuals of *S.* (*S.*) *coriniensis* sp. nov. were collected by hand from the walls and floor in the innermost part of the Jamurka (Rnjakuša II) Pit, in places that were in complete darkness, with a high degree of humidity and the presence of trickling water.

***Spelaeobates* (*Spelaeobates*) *coriniensis nonveilleri* Ćurčić, Vesović, Vrbica & Rađa, ssp. nov.**

https://zoobank.org/D92C0413-A52D-45A8-AE12-759986CD2F8F
Figs 4–6

Type material. ***Holotype*:** male (SSM) labeled as follows: "CROATIA, NORTHERN DALMATIA: city of Šibenik, island of Murter, settlement of Tisno, village of Jezera, Jezeranka Pit, 42 m a.s.l., 43°47'16.1"N, 15°37'25.3"E, 2.VI.2019, TR" (white label, printed) / "Holotypus *Spelaeobates* (*Spelaeobates*) *coriniensis nonveilleri* ssp. nov. Ćurčić, Vesović, Vrbica & Rađa det. 2022" (red label, printed).

Paratypes (10 specimens). The same data as for HT [three males and five females, IZFB]; two males (IZFB) labeled as follows: "CROATIA, NORTHERN DALMATIA: city of Šibenik, village of Banjevci, Šušnjevača Pit, 149 m a.s.l., 43°53'29.9"N, 15°38'20.8"E, 5.XI.2019, TR". All paratypes are labeled with white, printed locality labels and with red printed labels "Paratypus *Spelaeobates* (*Spelaeobates*) *coriniensis nonveilleri* ssp. nov. Ćurčić, Vesović, Vrbica & Rađa det. 2022" (Fig. 4).

Etymology. *Spelaeobates* (*Spelaeobates*) *coriniensis nonveilleri* ssp. nov. is named after late Prof. Dr Guido Nonveiller, a famous Serbian and Croatian biospeleologist and an excellent connoisseur of the subterranean beetle fauna of the Balkans.

Diagnosis. The new subspecies is morphologically closest to the nominotypic subspecies *S.* (*S.*) *coriniensis coriniensis* ssp. nov., with which we compared it.

Spelaeobates (*S.*) *coriniensis nonveilleri* ssp. nov. differs from *S.* (*S.*) *coriniensis coriniensis* ssp. nov. with respect to head shape (more elongate and narrower vs. shorter and wider), A6L/A3L (R 0.81–0.88 vs. R 0.88–1.00), shape of certain antennomeres (antennomeres I–V and IX–XI more elongate in first subpecies, whereas antennomeres VI–VIII more elongate in second subspecies), shape of pronotum (more elongate, widest before level of first third, lateral margins strongly convex anteriorly, with right hind angles vs. less elongate, widest at level of first third, lateral margins rounded anteriorly, with obtuse hind angles), PL/PW (R 1.24–1.38 vs. R 1.21–1.28), shape of process between mesocoxae on mesoventrite (sub-parallel vs. gradually narrowing apically), shape of elytra (more narrowed apically vs. less narrowed apically), shape of basal bulb

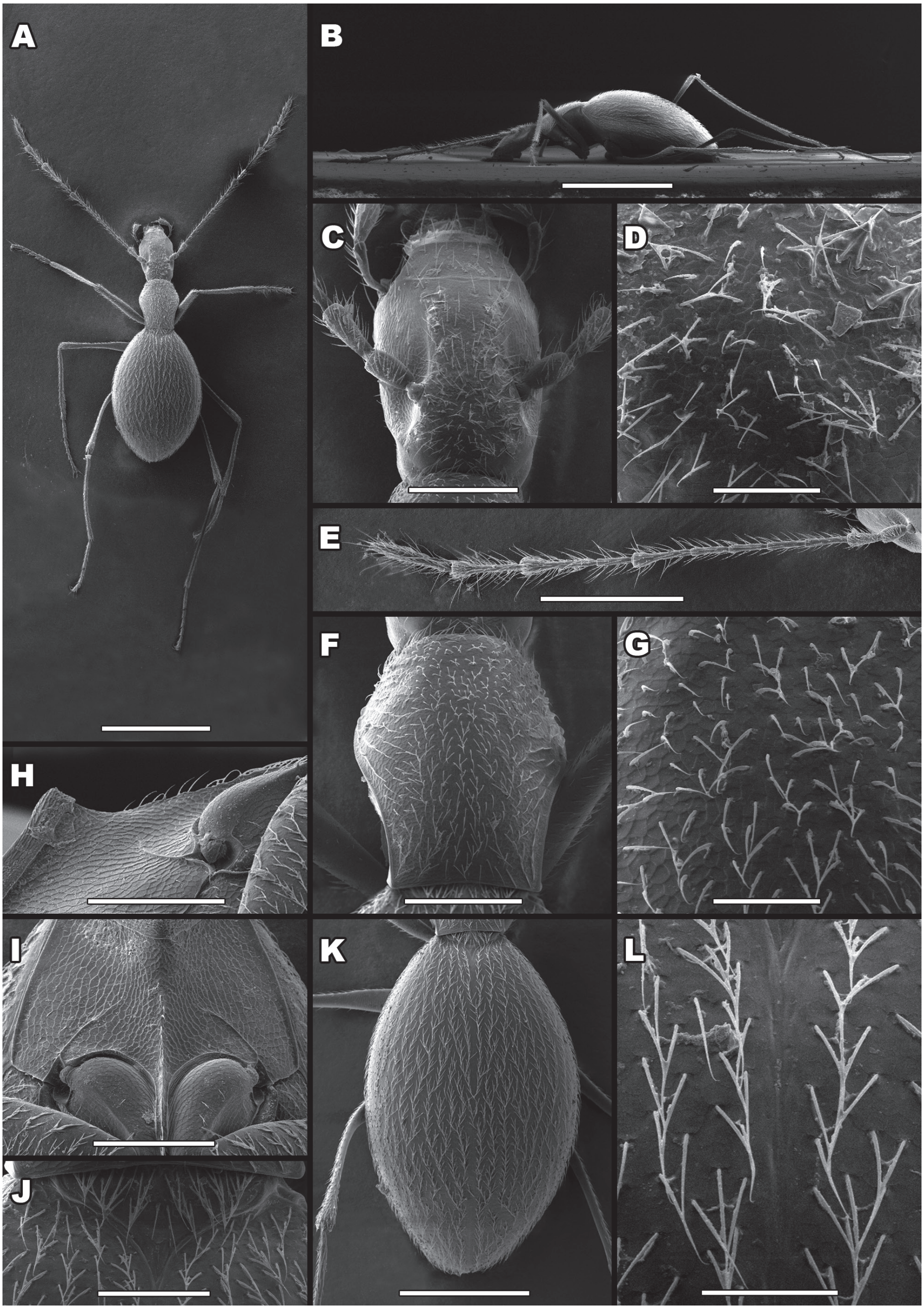

Figure 4. SEM images of the morphological structures of PT male (**A–G, J–L**) and PT female (**H, I**) of *Spelaeobates* (*Spelaeobates*) *coriniensis nonveilleri* ssp. nov. from the Jezeranka Pit, village of Jezera, settlement of Tisno, island of Murter, city of Šibenik, northern Dalmatia, Croatia **A** habitus, dorsal view **B** habitus, lateral view **C** head, dorsal view **D** microsculpture of head, dorsal view **E** right antenna, dorsal view **F** pronotum, dorsal view **G** microsculpture of pronotum, dorsal view **H** mesoventral carina, lateral view **I** mesoventrite, ventral view **J** scutellum, dorsal view **K** elytra, dorsal view **L** microsculpture of elytra, dorsal view. Scale bars: 1.0 mm (**A, B**); 0.5 mm (**E, K**); 0.2 mm (**C, F, H, I**); 0.1 mm (**J**); 0.05 mm (**D, G, L**).

in dorsal view (broadened distally vs. narrow, sub-parallel), and shape of spermatheca (less constricted medially vs. more constricted medially) (Table 1, Figs 2–6).

Description. Small-sized leptodirine. TL M 2.46 mm (2.42 mm in males, 2.52 mm in females), R 2.34–2.59 mm (2.34–2.52 mm in males, 2.47–2.59 mm in females).

***Habitus*:** Body shape leptodiroid (Fig. 4A, B), colour yellowish.

***Integument*:** Shiny, microsculptured both dorsally and ventrally (Fig. 4C, D, F–L). Sparsely distributed deep punctures present on head, while densely distributed, fine and separated on both pronotum and elytra (Fig. 4C, D, F, G, K, L). Entire body dorsally covered with yellow pubescence of short length (erect on head, while recumbent on both pronotum and elytra) (Fig. 4A, B).

***Head*:** More than one and a half times as long as wide (HL/HW M 1.57, R 1.53–1.61), with no differences in shape between males and females, anophthalmous, occipital carina in the shape of a curved concave line (Fig. 4A, C). Head widest in first quarter or between first quarter and third. Frons roundly impressed between antennal insertions. Labrum transverse, with a few long setae. First maxillary palpomere of similar length and width, shorter than second maxillary palpomere. Maxillary palpomeres II and III of similar length (M3L/M2L M 0.97, R 0.85–1.09). Penultimate maxillary palpomere widened apically. Last maxillary palpomere short, thin, gradually narrowing apically. Antennae inserted in basal quarter of head, slender, narrow proximally (except for first two antennomeres, which are widened), slightly widened distally, longer in males, AL M 1.86 mm, R 1.77–1.94 mm (1.86–1.94 mm in males, 1.77–1.85 mm in females), reaching end of elytra in males (Fig. 4A, B, E). Antennomeres I and II short and wide, second of which slightly longer and narrower. Following four antennomeres more slender and slightly longer than antennomere II. Antennomere III longer than adjacent antennomeres (A3L/A2L M 1.34, R 1.23–1.45; A3L/A4L M 1.21, R 1.07–1.31). Antennomeres VII, IX, and X quite dilated distally. Antennomere VIII relatively short and narrow, shorter and narrower than anatennomeres VII, IX, X, and XI. Ultimate antennomere thin, widened sub-distally, then narrowing apically, narrower than preceding (A11W/A10W M 0.84, R 0.67–1.00). Antennomere I shortest, while antennomeres IX and XI longest. Other ratios of length of certain antennomeres: A6L/A3L M 0.86, R 0.81–0.88; A8L/A3L M 0.75, R 0.65–0.81; A11L/A8L M 2.03, R 1.77–2.25.

***Prothorax*:** Pronotum bell-shaped, elongate, longer than wide (PL/PW M 1.30, R 1.24–1.38; M 1.29, R 1.24–1.33 in males; M 1.31, R 1.28–1.38 in females), widest between first fourth and third, wider (HW/PW M 0.91, R 0.87–0.97) and shorter than head (PL/HL M 0.91, R 0.89–0.96) (Fig. 4A, F). Lateral margins strongly convex anteriorly, then narrowing towards posterior end, markedly concave posteriorly. Pronotal base almost straight, slightly shorter than elytral base. PB/AM M 0.89, R 0.83–0.96. Anterior margin convex medially. Lateral margins and pronotal base rimmed. Fore pronotal angles weakly expressed, rounded, obtuse. Hind pronotal angles well-expressed, right, not protruding backwards. Pronotal disc moderately convex (Fig. 4B).

***Mesothorax*:** Mesoventral carina very low, barely noticeable, with a few setae (Fig. 4H). No tooth, anterior and posterior margins observed. Mesoventrite with a long, sub-parallel process between mesocoxae (Fig. 4I). Scutellum large, sub-triangular (Fig. 4K, J).

***Metathorax*:** Metaventrite with no carina.

***Elytra*:** Broad, ovoid, almost of same width in males and females (EL/EW M 1.63, R 1.55–1.76 in males; M 1.66, R 1.55–1.73 in females), markedly wider than pronotum (EW/PW M 2.28, R 2.18–2.36) (Fig. 4A, K). Maximum width a little before middle. Lateral margins arcuate. Marginal furrows not visible from above. Shoulders barely noticeable, obtuse, covered by hind pronotal angles. Elytral disc markedly convex, steeply declining basally and gently declining apically in lateral view (Fig. 4B). Parasutural stria absent. Elytral apex slightly attenuated, rounded. Pygidium covered by elytra.

***Legs*:** Elongate and thin (Fig. 4A, B). Femora broadened basally, constricted in distal half. Tibiae slender, gently curved, gradually widening distally. Each protibia with a very fine comb over entire apical third of outer margin. Fore tarsi four-segmented in both sexes, only first protarsomere in males slightly dilated (P1W/P2W M 1.20, R 1.00–1.50). Tarsal claws thin, elongate, curved, pointed apically.

***Male genitalia*:** Aedeagus elongate, thin, small, well chitinised, almost the same as in the nominotypic subspecies (Fig. 5A, B). Median lobe in dorsal view straight, gradually narrowing apically, with a sharp apex, barely longer than parameres (Fig. 5A). Median lobe in lateral view quite flattened, curved basally, almost straight proximally, narrowing apically (Fig. 5B). Basal bulb small, broadened distally and bilobed in dorsal view (Fig. 5A), elongate and broadened basally in lateral view (Fig. 5B). Tegmen wide from above (Fig. 5A), in the shape of a ring around basal bulb (Fig. 5B). Parameres elongate, thin, arcuate, sub-apically curved exteriorly, each with a moderately broadened rounded apex in dorsal view (Fig. 5A), while straight, sub-parallel in lateral view (Fig. 5B). Each paramera carrying four apical close-set setae, three of which longer, while one shorter (Fig. 5A). No copulatory piece observed within inner sac (Fig. 5A, B).

***Female genitalia*:** Spermatheca small, chitinised, curved, somewhat constricted medially, spherical both basally and apically (Fig. 6C). Gonostyli short, straight, moderately broadened, gradually narrowing distally, pointed apically. Each gonostylus with one long apical seta.

***Male abdominal sternite IX (urite)*:** Small, narrowing apically, sub-triangular (Fig. 6D).

***Female abdominal ventrite VIII*:** Small, transverse, with no anterior process, hairy, especially posteriorly (Fig. 6E).

Intrasubspecific variability. Some degree of intrasubspecific variability was noted in the new subspecies. It refers to the differences between the two known populations (one from the Jezeranka Pit, and the other from the Šušnjevača Pit). The following differences were observed between the individuals of the two populations mentioned: (i) head is widest in the first quarter in individuals from the population from the Jezeranka Pit vs. head is widest between the first quarter and third in specimens from the Šušnjevača Pit; (ii) antennomeres III, V, and IX, maxillary palpomere I, and elytra are more elongate in individuals from the Jezeranka Pit; (iii) antennomeres VI–VIII, X, and XI are more elongate in individuals from the Šušnjevača Pit; (iv) maxillary palpomere III is longer than maxillary palpomere II in individuals from the Šušnjevača Pit vs. maxillary palpomere III is shorter than maxillary palpomere II in individuals from

Figure 5. Bright-field images of the male genitalia of HT of *Spelaeobates* (*Spelaeobates*) *coriniensis nonveilleri* ssp. nov. from the Jezeranka Pit, village of Jezera, settlement of Tisno, island of Murter, city of Šibenik, northern Dalmatia, Croatia **A** aedeagus, dorsal view **B** aedeagus, lateral view. Scale bars: 0.1 mm.

the Jezeranka Pit. However, the identical shape of the aedeagus indicates that individuals from both populations belong to the same subspecies.

Sexual dimorphism. Some degree of sexual dimorphism was noted in this new subspecies. Namely, it was found that: (i) the females are slightly longer than the males; (ii) the antennae of the males are longer than those of the females; (iii) antennomeres VIII–X are more elongate in the males than in the females; (iv) first protarsomere is broader in the males than in the females.

Type locality. Jezeranka Pit, village of Jezera, close to the settlement of Tisno and the city of Šibenik, northern Dalmatia, Croatia.

Geographic distribution. This new subspecies is currently known from only two localities in the vicinity of the city of Šibenik, northern Dalmatia, Croatia – the Jezeranka Pit (type locality) in the village of Jezera, near the settlement of Tisno, and the Šušnjevača Pit in the village of Banjevci. The first site is on the island (Murter), while the second is on the mainland. At the same time, this is the second official finding of a species of the genus *Spelaeobates* on the mainland. It is likely that the new subspecies also lives at other insular and non-insular subterranean sites in the surrounding area in northern Dalmatia.

Figure 6. Bright-field images of certain morphological traits of *Spelaeobates* (*Spelaeobates*) *coriniensis coriniensis* ssp. nov. from the Jamurka (Rnjakuša II) Pit, village of Popovići, settlement of Gornji Karin, town of Benkovac, northern Dalmatia, Croatia (**A, B**) and *S.* (*S.*) *coriniensis nonveilleri* ssp. nov. from the Jezeranka Pit, village of Jezera, settlement of Tisno, island of Murter, city of Šibenik, northern Dalmatia, Croatia (**C–E**) **A, C** PT female, spermatheca, lateral view **B** PT female, gonostyli, dorsal view **D** HT male, abdominal sternite IX (urite) **E** PT female, abdominal ventrite VIII. Scale bars: 0.05 mm (**A, C**); 0.10 mm (**B, D, E**).

Bionomy and habitat. Specimens of *S.* (*S.*) *coriniensis nonveilleri* ssp. nov. were collected manually from the walls and floor in the innermost parts of the Jezeranka and Šušnjevača Pits, in places that were in complete darkness, with a high degree of humidity and the presence of trickling water.

***Spelaeobates* (*Spelaeobates*) *novaki* Müller, 1901**

Figs 7, 8

Type material. ***Topotypes*:** one male and four females (IZFB) labeled as follows: "CROATIA, NORTHERN DALMATIA: island of Dugi Otok, village of Savar, Strašna Peć Cave, 70 m a.s.l., 44°00'16.6"N, 15°02'19.1"E, 1.VII.1997, TR". All topotypes are labeled with white, printed locality labels (Fig. 7).

Remarks. For purpose of comparisons, we have examined the topotype material of *S.* (*S.*) *novaki* collected by the last author of this study. This species is described on the basis of the type series of specimens collected in September 1900 by Josef Müller and Petar Novak in two caves on two northern Dalmatian islands – the Strašna Peć Cave, village od Savar, island of Dugi Otok, and a small cave in the village of Mali Iž, island of Iž (Müller 1901). Later Pretner (1973) determined the correct name of the second cave site (its exact name is the Jezero Cave). In his original description of *S.* (*S.*) *novaki*, Müller (1901) did not indicate how many specimens are included in the type series, nor is there any information about their sex or where they were deposited. Based on the data and illustrations in the paper of Müller (1901), it can be concluded that the type series of this species consisted of both male and female specimens.

After reading both the original description of *S.* (*S.*) *novaki* by Müller (1901) and the subsequent morphological data on the species by Jeannel (1924), as well as a careful examination of the topotype specimens of *S.* (*S.*) *novaki*, we have found that some of the data given in the earlier literature on the morphology of the species do not agree with the characteristics of the topotype specimens we have observed. Namely, both Müller (1901) and Jeannel (1924) reported that the parameres of the aedeagus of *S.* (*S.*) *novaki* lack setae. Furthermore, in the drawing of the aedeagus by Müller (1901), no parameral setae are present. However, in the topotype male of *S.* (*S.*) *novaki*, we observed that each paramere has four apical setae, as in *S.* (*S.*) *coriniensis* sp. nov. In the work of Müller (1901), it was noted that the head of *S.* (*S.*) *novaki* is nearly twice as long as wide, the pronotum is 1.5 times longer than wide, antennomeres I and II are of similar width, the last antennomere is wider than the preceding ones, and the first protarsomere of the males is about twice as long as wide. However, in the specimens of the same species that we have examined, the head is about 1.5 times as long as wide, the pronotum is nearly one third longer than wide, antennomere I is wider than antennomere II, the last antennomere is narrower than the preceding ones, and the first protarsomere of the male is about 1⅔ times longer than wide (Table 1). Müller (1901) noted that the pronotum of *S.* (*S.*) *novaki* is finely punctate, but Jeannel (1924) reported that it is strongly punctate, which we also observed in our specimens. For these reasons, we have decided to redescribe the species *S.* (*S.*) *novaki* and add additional data on its morphology here.

Redescription. Small-sized leptodirine. TL M 2.63 mm (2.57 mm in males, 2.64 mm in females), R 2.57–2.70 mm (2.57 mm in males, 2.60–2.70 mm in females).

***Habitus*:** Body shape leptodiroid (Fig. 7A, B), colour yellowish-brown.

***Integument*:** Lustrous, microsculptured both dorsally and ventrally (Fig. 7C, E, F, H). Densely distributed deep punctures present on head, pronotum (often merged) and elytra (particularly strong) (Fig. 7C, E, F). Entire body dorsally covered with yellow pubescence of short length (erect on head, while recumbent on both pronotum and elytra) (Fig. 7A, B).

***Head*:** About one and a half times as long as wide (HL/HW M 1.47, R 1.38–1.53), slightly more elongate in males (HL/HW M 1.49 in males, M 1.46 in females), with no eyes, occipital carina in the shape of a curved concave line (Fig. 7A, C). Head widest between first third and half. Frons roundly impressed between antennal insertions. Labrum transverse, with a few long setae. First maxillary palpomere of similar length and width, shorter than second maxillary palpomere. Maxillary palpomeres II and III of similar length (M3L/M2L M 1.09, R 1.00–1.18). Penultimate maxillary palpomere widened apically. Ultimate maxillary palpomere

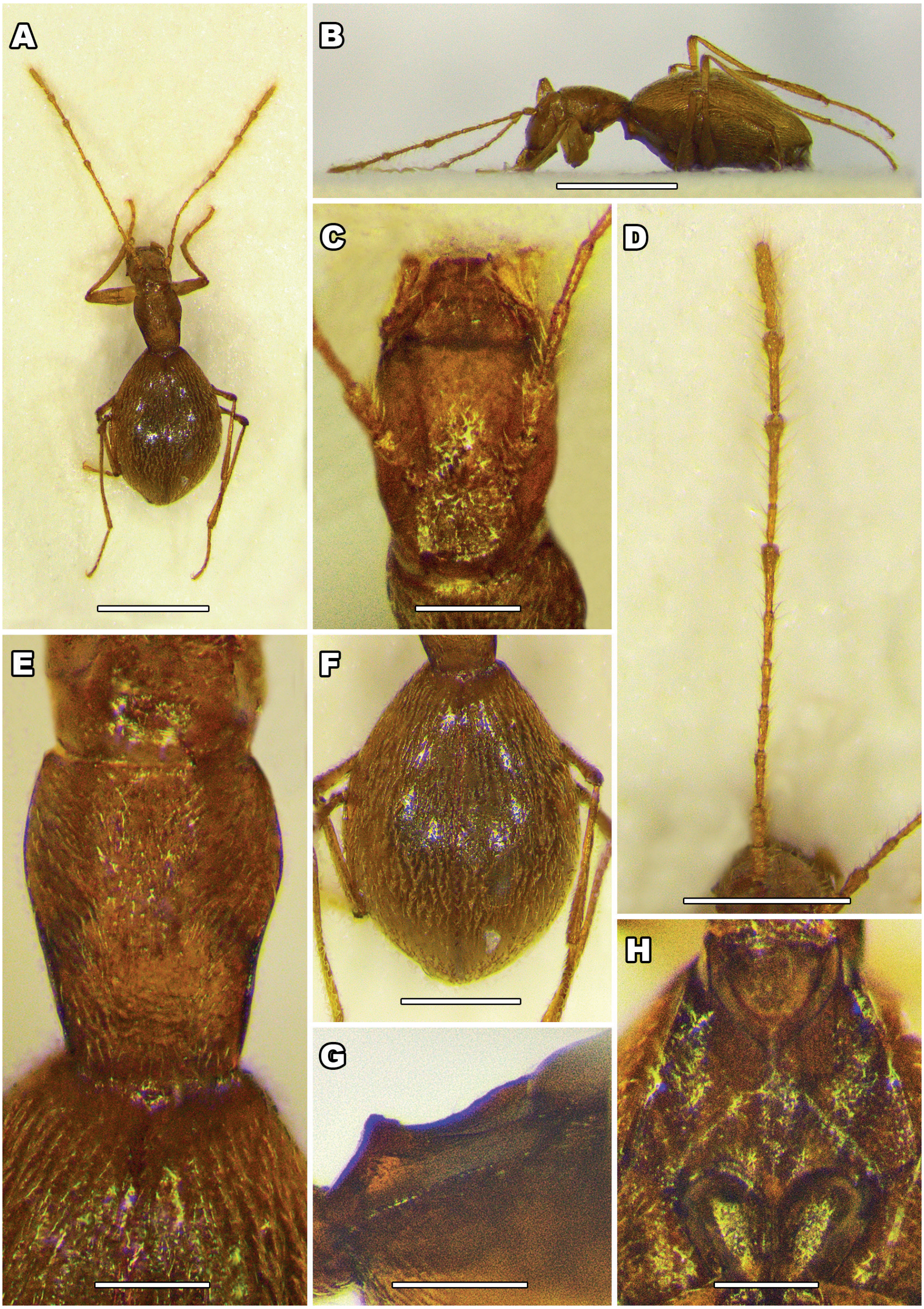

Figure 7. Bright-field images of the morphological structures of topotype male (**A–G**) and topotype female (**H**) of *Spelaeobates* (*Spelaeobates*) *novaki* from the Strašna Peć Cave, village of Savar, island of Dugi Otok, northern Dalmatia, Croatia **A** habitus, dorsal view **B** habitus, lateral view **C** head, dorsal view **D** left antenna, dorsal view **E** pronotum and scutellum, dorsal view **F** elytra, dorsal view **G** mesoventral carina, lateral view **H** mesoventrite, ventral view. Scale bars: 1.0 mm (**A, B**); 0.5 mm (**D, F**); 0.25 mm (**C, G**); 0.2 mm (**E, H**).

short, slender, gradually narrowing apically. Antennae inserted in basal quarter of head, thin, narrow proximally (except for first two antennomeres, which are thickened), slightly widened distally, longer in males, AL M 1.81 mm, R 1.75–1.93 mm (1.93 mm in males, 1.75–1.79 mm in females), not reaching end of elytra in both sexes (Fig. 7A, B, D). Antennomeres I and II short and wide, of similar length, second of which slightly narrower. Following four antennomeres thinner and slightly longer than antennomere II. Antennomere III longer than adjacent antennomeres (A3L/A2L M 1.37, R 1.23–1.42; A3L/A4L M 1.24, R 1.21–1.31). Antennomeres VII, IX, and X quite expanded distally. Antennomere VIII relatively short and narrow, shorter and narrower than anatennomeres VII, IX, X, and XI. Ultimate antennomere slender, widened sub-distally, then narrowing apically, narrower than penultimate one (A11W/A10W M 0.75, R 0.67–0.83). Antennomere VIII shortest, while antennomeres IX and XI longest. Other ratios of length of certain antennomeres: A6L/A3L M 0.79, R 0.76–0.82; A8L/A3L M 0.65, R 0.59–0.71; A11L/A8L M 2.27, R 2.09–2.40.

***Prothorax*:** Pronotum bell-shaped, elongate, longer than wide (PL/PW M 1.29, R 1.26–1.32; M 1.32, R 1.32 in males; M 1.28, R 1.26–1.30 in females), widest slightly before anterior third, broader (HW/PW M 0.93, R 0.88–0.98) and shorter than head (PL/HL M 0.96, R 0.90–0.98) (Fig. 7A, E). Lateral margins rounded anteriorly, after which they constrict towards posterior end, slightly concave posteriorly. Pronotal base straight, somewhat shorter than elytral base. PB/AM M 0.86, R 0.81–0.90. Anterior margin straight. Lateral margins and pronotal base rimmed. Fore pronotal angles weakly expressed, rounded, obtuse. Hind pronotal angles well-expressed, obtuse, not protruding backwards. Pronotal disc moderately convex (Fig. 7B).

***Mesothorax*:** Mesoventral carina very low, barely noticeable, with a few setae (Fig. 7G). No tooth, anterior and posterior margins observed. Mesoventrite with a long, sub-parallel process between mesocoxae (Fig. 7H). Scutellum large, sub-triangular (Fig. 7E, F).

***Metathorax*:** Metaventrite without carina.

***Elytra*:** Wide, ovoid, of similar width in males and females (EL/EW M 1.53, R 1.53 in males; M 1.55, R 1.49–1.60 in females), markedly wider than pronotum (EW/PW M 2.44, R 2.37–2.50) (Fig. 7A, F). Maximum width a little before middle. Lateral margins arcuate. Marginal furrows not visible from above. Shoulders barely visible, obtuse, covered by hind pronotal angles. Elytral disc markedly convex, steeply declining both basally and apically in lateral view (Fig. 7B). Parasutural stria absent. Elytral apex slightly attenuated, rounded. Pygidium not entirely covered by elytra.

***Legs*:** Elongate and slender (Fig. 7A, B). Femora widened basally, constricted in distal half. Tibiae thin, gently curved, gradually widening distally. Each protibia with a very fine comb over entire apical third of outer margin. Fore tarsi four-segmented in both sexes, only first protarsomere in males dilated (P1W/P2W M 1.67, R 1.67). Tarsal claws thin, elongate, curved, pointed apically.

***Male genitalia*:** Aedeagus elongate, slender, small, well chitinised (Fig. 8A, B). Median lobe in dorsal view straight, sub-parallel, gradually narrowing distally, with

Figure 8. Bright-field images of certain morphological traits of topotype male (**A, B**) and topotype female (**C, D**) of *Spelaeobates* (*Spelaeobates*) *novaki* from the Strašna Peć Cave, village of Savar, island of Dugi Otok, northern Dalmatia, Croatia **A** aedeagus, dorsal view **B** aedeagus, lateral view **C** spermatheca, lateral view **D** abdominal ventrite VIII. Scale bars: 0.2 mm (**A, B, D**); 0.1 mm (**C**).

a rounded apex, longer than parameres (Fig. 8A). Median lobe in lateral view quite flattened, curved basally, straight proximally and slightly bent downward distally, narrowing apically (Fig. 8B). Basal bulb small, narrow, sub-parallel and slightly widened distally in dorsal view (Fig. 8A), while elongate and widened basally in lateral view (Fig. 8B). Tegmen wide from above (Fig. 8A), in the shape of a ring around basal bulb (Fig. 8B). Parameres elongate, slender, arcuate, sub-apically curved exteriorly, each with a moderately widened rounded apex in dorsal view (Fig. 8A), while almost straight, sub-parallel in lateral view (Fig. 8B). Each paramera bearing four apical close-set setae, two of which longer, while two shorter (Fig. 8A, B). No copulatory piece observed within inner sac (Fig. 8A, B).

***Female genitalia*:** Spermatheca small, chitinised, straight basally, curved sub-apically, spherical both basally and apically (Fig. 8C). Gonostyli short, straight, moderately widened, gradually narrowing distally, pointed apically. Each gonostylus carrying one long apical seta.

***Male abdominal sternite IX (urite)*:** Small, narrowing apically, sub-triangular.

***Female abdominal ventrite VIII*:** Small, transverse, with no anterior process, hairy, especially posteriorly, slightly bilobed distally (Fig. 8D).

Geographic distribution. This species inhabits two caves located on two northern Dalmatian islands – the Strašna Peć Cave (island of Dugi Otok) and the Jezero Cave (island of Iž) (Pretner 1973). It is possible that it also inhabits other subterranean sites on the same and neighbouring islands.

Key to the taxa of the genus *Spelaeobates* [modified after Jeannel (1924)]

1 First protarsomere in males dilated, median lobe of aedeagus attenuated apically [*Spelaeobates* Müller, 1901] **2**

– First protarsomere in males narrow, median lobe of aedeagus bulging and club-like apically [*Pretneriella* V. Guéorguiev, 1976] **4**

2 Punctuation on pronotum strong, with merged punctures, elytra less elongate, median lobe of aedeagus sub-parallel, rounded apically .... ***S.* (*S.*) *novaki* Müller, 1901**

– Punctuation on pronotum fine, with separated punctures, elytra more elongate, median lobe of aedeagus narrowing distally, pointed at apex [*S.* (*S.*) *coriniensis* sp. nov.] **3**

3 Head shorter and wider, lateral pronotal margins rounded anteriorly, hind pronotal angles obtuse, elytra less narrowed apically, protarsomere I in males wider, basal bulb narrow, sub-parallel in dorsal view .... ***S.* (*S.*) *coriniensis coriniensis* ssp. nov.**

– Head more elongate and narrower, lateral pronotal margins strongly convex anteriorly, hind pronotal angles right, elytra more narrowed apically, protarsomere I in males narrower, basal bulb broadened distally in dorsal view....... .... ***S.* (*S.*) *coriniensis nonveilleri* ssp. nov.**

4 Lateral margins of pronotum entirely rimmed, mesoventral carina high and toothed **5**

– Lateral margins of pronotum rimmed only basally, mesoventral carina not toothed 7

5 Pronotum short, regularly narrowed and slightly sinuate basally, antennomere II not longer than antennomere I, elytra more convex, TL 2.6–2.8 mm [*S.* (*P.*) *pharensis* Müller, 1901] **6**

– Pronotum elongate, abruptly and deeply sinuate basally, antennomere II distinctly longer than antennomere I, elytra less convex, flattened in sutural region, TL 2.8–3.0 mm .... ***S.* (*P.*) *czernyi* Breit, 1913**

6 Smaller, pronotum less elongate, with maximum width less forward, wider basally, punctuation and pubescence of elytra a little less dense .... ***S.* (*P.*) *pharensis pharensis* Müller, 1901**

– Larger, pronotum more elongate, with maximum width moved further forward, narrower basally, punctuation and pubescence of elytra a little denser .... ***S.* (*P.*) *pharensis langhofferi* Müller, 1931**

7 Punctuation on pronotum deep and strong, TL more than 2.8 mm .... ***S.* (*P.*) *kraussi* Müller, 1903**

– Punctuation on pronotum superficial and fine, TL less than 2.8 mm **8**

8 Pronotum shorter, more rounded anteriorly, more deeply sinuate posteriorly, TL 2.7 mm .... ***S.* (*P.*) *bachofeni* Breit, 1913**

– Pronotum more elongate, less rounded anteriorly, less deeply sinuate posteriorly, TL 2.8 mm .... ***S.* (*P.*) *peneckei* Müller, 1903**

New findings of the genus *Spelaeobates*

We had at our disposal as comparative material two samples of the genus *Spelaeobates*, collected on two islands in central Dalmatia by the last author of this study, which belong to the taxa *S.* (*P.*) *kraussi* and *S.* (*P.*) *pharensis langhofferi* (see the chapter Other examined taxa). A sample of *S.* (*P.*) *kraussi* (one male and one female) was collected last year in the Vičja Jama Pit (Vidova Gora peak, village of Nerežišća) on the island of Brač, while a sample of *S.* (*P.*) *pharensis langhofferi* (two females) was collected 10 years ago in the Jama na Boroviku Pit (village of Pitve, near the town of Jelsa) on the island of Hvar. Both taxa have been recorded so far only from their type locality – *S.* (*P.*) *kraussi* from the Dobra Jama Pit, Vidova Gora peak, village of Nerežišća, island of Brač, while *S.* (*P.*) *pharensis langhofferi* from the Kruščica Cave, near the town of Stari Grad, island of Hvar. We report herein the first findings of these two taxa outside their type locality.

Discussion

Our new findings of the genus *Spelaeobates* were completely surprising considering that more than a century has passed since the last species of this genus was described (Perreau 2000, 2015; Hlaváč et al. 2017). The taxa of this genus are very rare, which is indicated by a small number of species that have been described so far, despite the fact that detailed biospeleological explorations have been carried out in Dalmatia so far (Pretner 1973). Future biospeleological research should be intensified in northern Dalmatia (both on the islands and on the mainland), where additional new *Spelaeobates* species and subspecies to science could be expected.

It was thought for a long time that the genus *Spelaeobates* is endemic to the Adriatic islands, which belong to northern and central Dalmatia (Croatia). Namely, at the beginning of the 20th century, several new species of this genus to science were described, inhabiting a total of 17 subterranean localities (caves and pits) on the islands of Iž, Dugi Otok, Hvar, Brač, and Vis (Pretner 1973). Jalžić (1982) reported the first finding of this genus on the mainland. He found three specimens of *Spelaeobates* on bat guano in the Golubnjača Cave in the village of Kaštel Žegarski near the town of Obrovac in northern Dalmatia (Croatia) (Jalžić 1982). This taxon was not identified to the species level due to the small number of specimens available to Jalžić. Perhaps these specimens might belong to the nominotypic subspecies of *S.* (*S.*) *coriniensis* sp. nov., inhabiting the Jamurka (Rnjakuša II) Pit, which is located in the vicinity, too. The species from the Golubnjača Cave might belong to the subgenus *Spelaeobates*, considering that the two new taxa of this subgenus to science described in the current study are distributed in the surrounding area. One of the priorities is to collect additional *Spelaeobates* specimens (including males) from the Golubnjača Cave, which would make it possible to determine whether they belong to any of the known taxa. A comprehensive molecular study of all taxa of this genus would be helpful to determine their definitive phylogenetic relationships and taxonomic status.

Our study confirms that the insular genus *Spelaeobates* is also distributed on the mainland, where it inhabits a relatively wide area in northern Dalmatia, and its distribution range is probably even broader. Our findings of two new *Spelaeobates* taxa to science [one species from the Jamurka (Rnjakuša II) Pit and one subspecies from the Jezeranka and Šušnjevača Pits] are the first accurate findings of a species of this genus on the mainland. It is interesting to point out that the subspecies *S.* (*S.*) *coriniensis nonveilleri* ssp. nov. inhabits both the island of Murter and the nearby mainland (admittedly, this island is by far the closest to the mainland compared to all the other islands where taxa of the genus *Spelaeobates* have been found).

All taxa of the genus *Spelaeobates* are distributed in the proximity of the Adriatic Sea, whether recorded on the islands or on the nearby mainland. Also, subterranean sites where the members of this genus were found are located at low altitudes (42–216 m a.s.l. in the case of the locations where two new taxa of *Spelaeobates* to science were found).

Based on the presence of the first dilated protarsomere in males and the apically narrowed median lobe of the aedeagus, we classified both new taxa to science (a new species and a new subspecies) in the subgenus *Spelaeobates* s. str. In contrast, species of the subgenus *Pretneriella* have a narrow first protarsomere in males, as well as a bulging median lobe of the aedeagus, which is club-like apically (Jeannel 1924; Guéorguiev 1976). Also, both new taxa are geographically closer to *S.* (*S.*) *novaki*, the only species previously belonging to *Spelaeobates* s. str., than to species of the subgenus *Pretneriella* (Fig. 1). The definitive status of new and other taxa within the genus *Spelaeobates* and its subgenera will be resolved by studying a larger number of taxa, preferably using molecular techniques.

Intrasubspecific variability was found between two recorded populations of *S.* (*S.*) *coriniensis nonveilleri* ssp. nov. – one from the Jezeranka Pit and the other from the Šušnjevača Pit. In addition, morphological differences related to sexual dimorphism were noted in both new taxa. Females of both taxa were expected to be larger and have broader elytra compared to males (Jeannel 1924; Ćurčić et al. 2021). Interestingly, no differences in body length and elytral width between sexes were observed in *S.* (*S.*) *coriniensis coriniensis* ssp. nov. In addition, no differences in the shape of the head and pronotum and elytral width between males and females were detected in *S.* (*S.*) *coriniensis nonveilleri* ssp. nov.

During geotectonic events, the Adriatic microplate was pulled into orogenic processes, which formed the Dinarides. The island relief of the northeastern part of the Adriatic Sea was created in post-Pleistocene by rising sea level 100 m and it geotectonically belongs to the Outer Dinarides. The Adriatic archipelago is an inseparable part of the orogenic mountain system of the Dinarides and a connection of islands and mountains is visible due to its equal extension in northwest-southeast direction (Bognar 1999). Thirteen thousand years ago, the Adriatic Sea was a large valley with many mountains. Today, the peaks of the mountains and the hills represent the islands and the coast. The Adriatic Sea was formed by large post-glacial floods. Cycles of drying and flooding shaped small biogeographical units. The Messinian salinity crisis, the Zanclean flood, the sea-level decrease during the last glacial period, and the ultimate flood in the Adriatic basin as a consequence of ice melting that began 18,000 years ago may explain the distributional patterns of species in the Adriatic basin (Van Straaten 1970; Maselli et al. 2014; Pellegrini et al. 2018), including the taxa of the genus *Spelaeobates*.

The fact that the territory of the former Yugoslavia, Bulgaria, and northern Greece was part of North Aegeis during most of the Tertiary indicates that the majority of terrestrial Balkan troglobites, including most of the endemic genera of the tribe Leptodirini, are of North Aegeid origin. This also applies to most representatives of the subtribes Bathysciotina and Leptodirina, as well as to all genera of the subtribes Anthroherponina and Spelaeobatina, including *Spelaeobates*. As most Balkan subterranean taxa of the tribe Leptodirini, two new *Spelaeobates* taxa to science described in the current study are also typical North Aegeid relicts, as evidenced by their location north of the Trans-Aegean fault (Popov et al. 2004).

The palaeokarst in the area of Dalmatia is very old (Gaudenyi and Jovanović 2012) and it is likely that the karstic areas of Dalmatian islands were once connected to karstic regions that now belong to the mainland of Dalmatia, which could favor the connection of their subterranean faunas in old geological times.

All previous authors agree that the genus *Spelaeobates* has a specific phylogenetic position within the tribe Leptodirini. Jeannel (1924) classified it in the phyletic series of *Spelaeobates*, while Guéorguiev (1974, 1977) established a separate subtribe Spelaeobatina, which contains only the genus *Spelaeobates*. Although the position of the antennal insertions (in the last quarter of the head) and the shape of the claws of the legs (enlarged and vertically blunt) indicated that Anthroherponina and Spelaeobatina are closely related subtribes (Jeannel 1924; Guéorguiev 1974, 1977), the prevailing opinion is that Spelaeobatina is actually closer to Bathysciina than to Anthroherponina (Perreau and Pavićević 2008; Njunjić et al. 2017), based on the analysis conducted by Casale et al. (1991). Thus, it can be assumed that the subtribes Spelaeobatina and Anthroherponina are not monophyletic since the phylogenetic significance of the position of the antennal insertions is debatable (Perreau and Pavićević 2008).

The genus *Prospelaeobates* Giachino & Etonti, 1996, which inhabits subterranean sites in southwestern Slovenia and northern Dalmatia (island of Cres, Croatia) (Perreau 2000, 2015), was thought to be related to *Spelaeobates* based on the presence of four tarsomeres in males and the structure of the aedeagus (Giachino and Etonti 1996). However, in the genus *Prospelaeobates*, the mesocoxal cavities are separated by an intercoxal process, which extends to the anterior margin of the metathorax (Giachino and Etonti 1996), in contrast to the genus *Spelaeobates*, where the mesocoxal cavities are fused (Giachino and Etonti 1995). Although originally considered related to *Spelaeobates* (Giachino and Etonti 1996), *Prospelaeobates* was later transferred to the subtribe Bathysciina (Newton 1998; Polak and Bognolo 2003).

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Eye convergence is evoked during larval prey capture (LPC) without visual stimulus and in blind cavefish

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Abstract

In zebrafish larvae, the first response when detecting prey is an oculomotor behavior; eye convergence. Eye convergence increases the overlap between the visual fields of the left and right eyes to prepare for tracking prey. A high vergence angle is maintained throughout the prey-tracking and capture swim phases, enhancing binocular depth. Since the discovery of eye convergence, hundreds of articles reporting on this behavior in zebrafish have been published. In this study, we found that the larvae of blind tetra cavefish, *Astyanax mexicanus*, despite being adapted to the absence of visual stimuli due to the lack of light in the cave, have retained the oculomotor behavior of eye convergence in their vestigial eyes. In *Astyanax*, eye convergence responses can be triggered singlehandedly by vibrations elicited with a glass rod at frequencies similar to those generated by its prey (10–35 Hz). The blind cave tetra offers an intriguing combination of regression of the eye structure, while retaining several of the physiological functions and actions performed in the eye, including light-entrained retinomotor rhythms and eye convergence.

Keywords

Astyanax, behavior, binocular vision, eye convergence, larval prey capture, ocular vergence, troglobite

Introduction

Darwin (1872) recognized descent with modifications during evolution by observing remnant structures, such as the reduced wings of flightless birds, the hind limb remnants of pythons, and the degenerate eyes of blind cave organisms. A vestigial structure is part of an organism that has diminished in size during its evolution because the function it served decreased in importance or became unnecessary (Bergman 2000). The regressive phenotypes of cave animals puzzled Darwin, who famously remarked, "As it is difficult to imagine that eyes, though useless, could in any way be injurious to animals living in darkness, I attribute their loss solely to disuse."

Often, natural selection cannot eliminate vestigial structures because they have retained some essential function. For example, the human embryo has gills slits like a fish. Why are the gill slits retained? During development, these gills become various structures essential for survival, such as the ear cavities, middle ear bones, muscles for chewing, the lower jaw, and certain parts of the neck including the thymus and thyroid (Manley and Capecchi 1998). Likewise, vestigial structures can acquire new functions. For instance, male pythons have little, claw-like structures derived from the hindlimbs of their four-legged ancestry. These vestigial structures now aid with courtship (Bejder and Hall 2002).

Cave animals are excellent models which can provide insight into the general principles of regressive evolution. Many organisms in caves retain features that can no longer serve their ancestral purpose. For example, the Cholevid beetle, *Ptomaphagus hirtus*, common in Mammoth Cave, Kentucky, has tiny vestigial eyes that retain light perception and have a complete circadian clock gene network (Friedrich et al. 2011). Likewise, the cave amphipod, *Stygobromus allegheniensis*, from the Ice caves in N.Y., follows circadian rhythms albeit modified (Espinasa et al. 2016). In the family Gonyleptidae, cave-dwelling opilionid species possess elongated appendages but retain some of the pigmented layer and the eye's lens (Pérez and Kury 2002).

Studies of regressed structures generally assume them to be nonfunctional (Fong et al. 1995). However, and in following with Darwin's statement that something useless may not necessarily be in any way injurious to animals, Espinasa and Jeffery (2006) questioned whether the loss of the physiological function and the cessation of the actions performed by an organ is a prerequisite for the structural degeneration of an organ. The blind cave tetra, *Astyanax mexicanus* offers an intriguing combination of regression of the structure of the eyes, while retaining some of the physiological functions and actions performed in the eye (Espinasa and Jeffery 2006); In teleosts living in surface habitats, during the day the cells in the retinal pigmented epithelium (RPE) disperse their pigmented granules, shielding photoreceptors from excessive light (King-Smith et al. 1996). At night, the situation is reversed and exposed photoreceptor can catch the maximum amount of available photons. Light and an endogenous circadian rhythm regulates the movement of cones and rods (Burnside 2001). In the blind tetra, fry retain the capacity to exhibit light-entrained retinomotor rhythms that move their retinal pigmented epithelium (Espinasa and Jeffery 2006). These retinomotor rhythms would appear to have no function in blind cavefish. Movements of an unpigmented RPE would not reduce the extent of bleaching of photoreceptors, which are essentially absent in cavefish. Likewise, maintenance of retinomotor activity would not be expected to optimize visual capabilities in cavefish, which do not experience visual stimuli in the cave, nor a daily circadian rhythm is expected in a habitat that does not experience light differences between day and night.

Adult *Astyanax* cavefish have minute optic capsules buried deep beneath the integument, which are not responsive to visual stimuli (Voneida and Sligar 1976; Voneida and Fish 1984). Surface fish have large eyes (Fig. 1A, B). Despite the absence of functional eyes in adult cavefish, eye primordia, including the lens vesicle, the optic cup, most of the retina, and the RPE are initially formed in embryos (Yamamoto and Jeffery 2000). This is consistent with both surface and cavefish fry showing strong adverse reactions when exposed to intense light. This reaction persists for a few days after birth. For a short period, a normal-appearing retina, including ganglion, bipolar, horizontal, and amacrine cells, is present (Jeffery et al. 2000). The first sign of eye degeneration occurs at 1.5 days postfertilization (dpf) when the lens begins to undergo apoptosis (Jeffery and Martasian 1998; Yamamoto and Jeffery 2000), and by 2 dpf retinal degeneration can also be detected (Langecker et al. 1993). Subsequently, eye growth arrests Retinal and RPE organization is gradually disrupted. By 10 dpf, only a few cells containing rhodopsin mRNA are found in the cavefish retina at this stage (Strickler and Jeffery 2009). By two weeks (Fig. 1D and F), cones are essentially absent in Pachón cavefish (Espinasa and Jeffery 2006). Eventually, the degenerating eye sinks into the orbit and is covered by an integument.

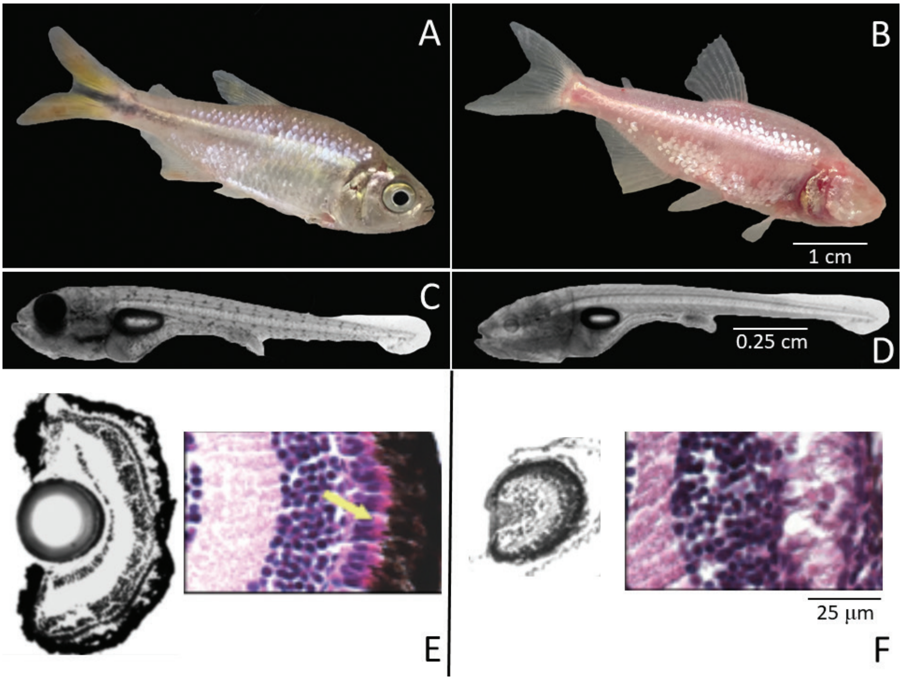

Figure 1. *Astyanax mexicanus* has two morphs; a surface, eyed morph (**A**) and a blind, cave morph (**B**) whose nonfunctional optic capsules are buried deep beneath the integument. At birth, cavefish have eyes and respond to light, but soon after, the eye degenerates. At 16–21 dpf, both the surface (**C**) and the cavefish larvae (**D**) have eyes. However, while the eye capsule and retina of the surface larvae (**E**) are well suited for vision, the eye capsule of the cavefish (**F**) has degenerated, the lens has undergone apoptosis, the outer nuclear layer of the retina is not completely differentiated, and there are essentially no photoreceptors. Cavefish larvae may detect light and darkness at this stage, but they lack central visual acuity and are thus blind to form perception. The scale in the right column is the same for the left column.

For this study, we concentrated on another physiological function and actions performed by the eyes of fish; eye convergence during Larval Prey Capture (LPC) behavior. LPC is characterized by a fast-striking motion toward the prey within tens of milliseconds. Serial time-lapse images of single prey capture events have revealed that in *Astyanax* (Espinasa et al. in press), when visual stimuli are available, they strike mostly at a target directly in front of them, bending the most caudal region of their tail (J turn). Conversely, surface fish under dark conditions and cavefish in both light and dark conditions strike mostly at prey on their side, using a C-bend turn. In zebrafish, distinct sensory inputs activate different neural circuits that result in C and J turns (Liu and Fetcho 1999; Fajardo et al. 2013). The most significant difference between the two morphs of *Astyanax* is that strike distance is significantly greater in cavefish compared to surface fish, suggesting cavefish may have improved their ability to detect prey in the dark (Lloyd et al. 2018). The expression of LPC in fry, and the Vibration Attraction Behavior (VAB) for surface fish is symmetric, with fish striking or examining prey equally on both sides. For cavefish fry or adults in the field, it is different among cave populations, with Pachón's "handedness" preferentially striking or examining with the right side of their head while the Tinaja, Sabinos, Molino and Toro cave populations preferentially using their left side (Espinasa et al. 2022; Espinasa et al. in press). These authors proposed that if there is an adaptative effect for asymmetric sensitivity, it selects for asymmetry itself. Not necessarily for the side being specialized.

Analysis of zebrafish conducting LPC while hunting paramecia uncovered a novel oculomotor behavior, eye convergence, which constitutes the first response of larvae to their prey (Bianco et al. 2011). In the study, prey or visual stimuli such as moving dots causes converging eye movements and a J-turn of the tail. These became the defining characteristics of a zebrafish's natural hunt. Eye convergence will likely increase the overlap between the visual fields of the left and right eyes to prepare for tracking prey. A high vergence angle is maintained throughout the prey-tracking and capture swim phases, enhancing binocular depth (Bianco et al. 2011). Since Bianco's et al. discovery of eye convergence, hundreds of articles reporting on this behavior have been published, many of them to understand neural networks.

This study aims to establish if fry from cavefish, despite being adapted to living in an environment characterized by perpetual darkness, have retained eye convergence when conducting LPC.

Methods

Fish rearing and maintenance

For this study we used the recordings of LPC used by Espinasa et al. (In press). For that study, animal husbandry was carried out as previously described (Borowsky 2008). Most work was conducted at the German Sumbre laboratory at the Institut de Biologie de l'ENS (IBENS), CNRS, France. All experiments performed at German Sumbre laboratory were approved by Le Comité d'Éthique pour l'Expérimentation Animale Charles Darwin (APAFIS#27495-2020100614519712 v14). Some specimens originated from Sylvie Rétaux at the Paris-Saclay Institute of Neuroscience, CNRS and University Paris-Saclay, France laboratory. Sylvie Rétaux's authorization for the use of *Astyanax mexicanus* in research is 91–116. The animal facility of the Institute received authorization B91272108 from the Veterinary Services of Essonne, France, in 2021. Fish were housed at 21 °C ± 1 °C. Lights were kept on a 14:10 h light-dark cycle. All fry used for experiments were fed on live *Artemia* nauplii starting on the 6th dpf. This study used two populations: Pachón cave's, and a surface population derived from the Choy River. Descriptions of the cave and the surface locality can be found in Elliott (2018).

Artemia preparation

Approximately 24 hours before behavioral experiments, Brine shrimp cysts (*Artemia salina*) were added to a plastic container with 1.2 L of water at a salinity of 25–30 ppt, pH of 7.5–8.5, and a temperature of 28 °C, with constant aeration. Immediately prior to testing, *Artemia* were rinsed with fresh water and placed into recording chambers. Only newly hatched *Artemia* nauplii, of the 1st instar stage, were used in behavioral experiments to ensure consistency of vibrational stimuli.

Recording of larval prey capture (LPC) behavior

As mentioned before, recordings of LPC were the same as the ones used by Espinasa et al. (In press). To get enhanced amplification and finer resolution, some new recordings were done with a Baumer camera attached to a microscope, plus some new recording with an iPhone 12 Mini, iOS version 15.5, attached to a tripod. These recordings were 1080p HD.

For recordings of LPC behavior on live prey, single fish were placed in a 9 cm diameter petri dish filled with ~20 mm of water to constrict the larvae into a single focal plane. Fry were allowed to acclimate for 2 minutes before the experiment began. Approximately 30 *Artemia* nauplii were used to record feeding behavior, and fish were imaged until they completed at least four successful strikes.

For recordings of LPC behavior on a vibrating glass rod, microinjection needles were made from glass capillaries with a Narishige's PC-10 Dual-Stage Glass Micropipette Puller. Borosilicate glass capillaries were heated and pulled to get fine needles, like those used for cell injection. The tip of the glass rod had a diameter of ~0.15 mm, about half the size of an *Artemia* nauplii. The vibration stimulus was generated using the ~0.15 mm diameter glass rod attached to an audio speaker (8ohm 0.1W 38 mm speaker) that produced 10 Hz with a TTI TG210 2MHz Function Generator. The peak-to-peak voltage was set to 21V. The axis of the vibration was in the horizontal plane. Individual fish were placed in a 9 cm diameter petri dish or a 3.5 cm diameter petri dish with water to a depth of ~3 mm. Fry acclimated in the experimental room for at least 2 hours. They were then transferred gently to the Petri dishes, where they further acclimated for 2 minutes before introducing the glass rod. The age of the fry tested was 16–21 dpf (Fig. 1C, D).

Quantification of eye convergence during LPC behavior

An analysis frame by frame of the recording was done starting 2 seconds before the initiation of movement toward the prey or vibrating glass rod until 2 seconds after LPC ended. Eye vergence angles were measured before, during, and after responses to the stimuli by drawing two lines along the width of each eye until the line from one eye converged with the line drawn from the other (Figs 2C and 3C). This allowed for predicting changes in the binocular visual field in response to the stimuli (Figs 2C vs. 2E and 3C vs. 3E).

A line was also drawn perpendicular to the eye's width, passing through the center of the pupil, in the direction of the center of that eye's visual field (Fig. 4A). This allowed for showing of the changes in the direction at which an eye is pointing in response to the stimuli (Figs 4A vs. 2D).

Results

Do surface *Astyanax* display eye convergence with non-visual stimuli?

Larval prey capture (LPC) behavior is characterized by a fast-striking motion toward the prey within tens of microseconds. Our first experiment tested if surface *Astyanax*

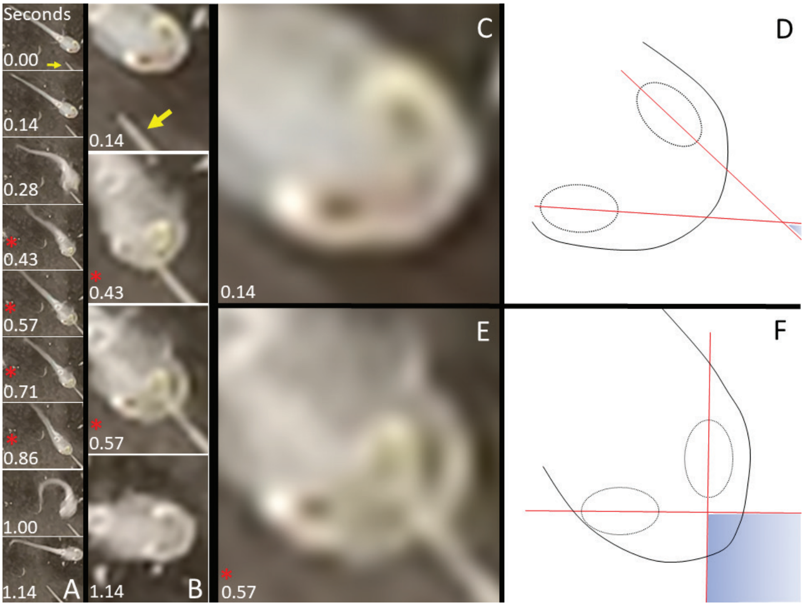

Figure 2. Eye vergence in surface fish stimulated by a vibrating (10 Hz) glass rod (**A, B**). Larval prey capture (LPC) behavior is characterized by a fast-striking motion toward the vibrating glass rod (yellow arrow) within tens of microseconds. Red asterisks highlight instances when the eyes converged. Higher magnification to highlight the changes in eye position during a strike (**C–F**). Freely swimming larvae have eyes pointing sub-perpendicular to their body in which the binocular overlap (blue) region of their visual space is minimal (**C, D**). During LPC, the mean eye vergence angle changes, largely expanding the binocular area of visual space (**E, F**).

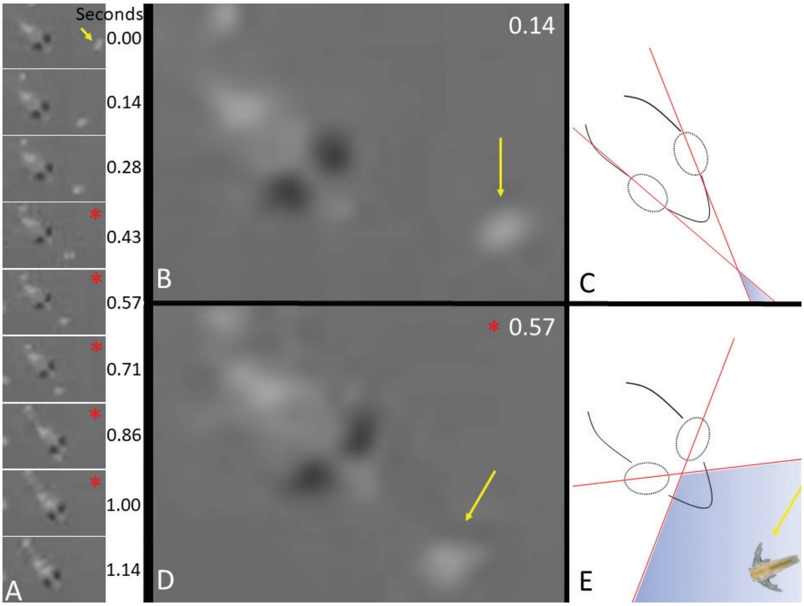

Figure 3. Eye vergence in surface fish while in the dark (**A**). Higher magnification to emphasize that despite being in the dark and without visual stimuli, the eyes change position (**B, D**) which, if illuminated, would have largely expanded the binocular proportion of visual space shown in blue (**C, E**). Notice that eyes converged when prey is detected at a distance (**A:0.43** and **D**), followed by a strike (**A:0.86**). Soon after, eyes return to normal position (**A:1.14**). Yellow arrows highlight the prey and red asterisks highlight instances when the eye converged.

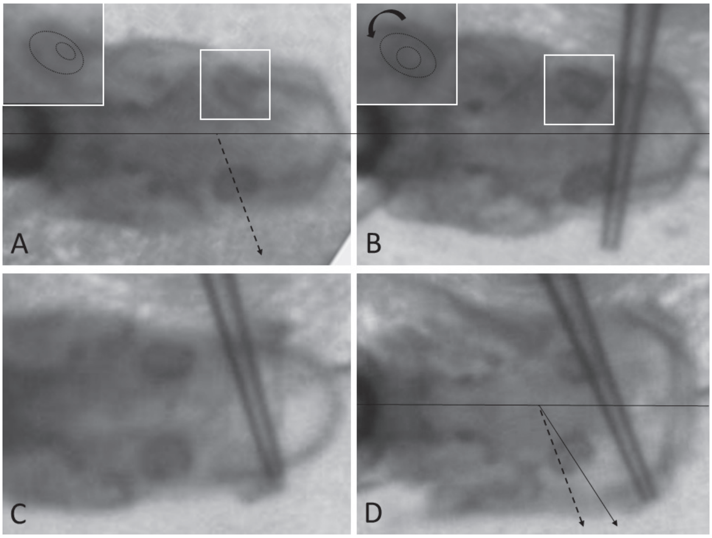

Figure 4. Blind cavefish *Astyanax* larvae have ocular vergence during LPC in response to vibrations from a glass rod, which elicits a strike behavior. Freely swimming larvae have eyes pointing sub-perpendicular to their body (**A**). When the source of a vibration stimulus is over the head, eyes turn upward (**B**). This was followed by a strike in which the cavefish larvae bit the glass rod (**C**). Eyes vergence remains for a few moments after a strike (**D**). Dotted arrows highlight eye angle before vergence to show the change of eye position.

larvae have ocular convergence during LPC when presented with a source of vibrations under light conditions that are not the stereotypical image of prey, such as a microcrustacean. For this, we used a vibrating glass rod at a frequency of 10 Hz. Ten Hz is a frequency similar to the one generated by *Artemia* nauplii that preferentially trigger successful strikes by *Astyanax* larvae (Espinasa et al. in press). We observed that, during striking episodes, larval surface fish converged their eyes (Fig. 2). Furthermore, the eyes were maintained at a high vergence angle throughout the LPC until soon after the release of the glass rod. Surface larval *Astyanax* seem to engage in a binocular viewing mode when hunting. Scale drawings showed predicted changes in the binocular visual field of larval *Astyanax* due to eye convergence (Fig. 2D versus 2F). Freely swimming larvae have eyes pointing sub-perpendicular to their body. As such, the region of binocular overlap (blue) of their visual space is minimal (Fig. 2D). During LPC, the mean vergence angle changed, advancing the binocular visual field to close to the front of the mid-point of the eyes, and largely expanding the binocular proportion of visual space.

Our second experiment tested if surface *Astyanax* larvae have ocular convergence during LPC with no visual stimuli. For this, we recorded LPC with an infrared LED light source. In trials, vergence of the eyes responded to the non-visual stimulus (Fig. 3). Both eyes could orient in the direction of the stimulus (Fig. 3A; 0.43–0.71 seconds), before the strike motion (Fig. 3A; 0.71–0.86 seconds). The first element of the behavioral response to the moving prey is a nasally-directed rotation of the eyes. C-bend turn movements or J-turns of the tail commence tens of a second later. Ocular convergence ended soon after the capture of the prey (Fig. 3A; 1.14 seconds). Despite being in the dark, convergent eye movements appeared to represent the first behavioral element in the hunting routine. Convergent eye movements can occur at the onset of the distinctive series of prey-tracking maneuvers in which larval fish reduce the distance and angular deviation between themselves and their prey. In surface *Astyanax* larvae, non-visual stimuli appear to activate LPC concurrently with ocular convergence. Of note, eye vergence started when the source of the stimuli was still far from the body. Direct contact with the body by the prey was not needed, and ~10 Hz vibrations, sound, smell, and/or other stimuli can activate the ocular vergence at a distance.

Do “blind” cave *Astyanax* display eye convergence?

As the introduction mentions, *Astyanax* cavefish and surface fish are initially born with equivalent eye structures, and both respond actively to light stimuli. While up to adulthood, cavefish may have some type of detection and response to light, cavefish larvae become effectively blind to patterns other than shadows early on. As reviewed in the introduction, in the 16–21 dpf cavefish larvae used for this study there is an overall degeneration of the eye capsule and almost complete regression of the outer nuclear layer that contains the cell bodies of the photoreceptor cells (Fig. 1E, F). It is assumed that while the “blind” cavefish larvae used for this study may detect light and darkness, they completely lack central visual acuity for form perception.

Our third experiment tested if blind cavefish *Astyanax* larvae have ocular vergence during LPC when presented exclusively with vibrations in the range generated by their prey. For this, we used a vibrating glass rod at a frequency of 10 Hz. Recordings for experiments showed that freely swimming cavefish larvae have eyes in a lateral-oriented position, but when a stimulus is above them, their eyes move upward, as reflected by the position of the pupil (Fig. 4A, B). Likewise, when the stimulus is in front of them, ocular convergence occurs with the center of the eye positioning forward (Fig. 4D). Despite their apparent blindness, the eye tracked the respective position of the source of vibrations with respect to the body of the fish (Fig. 5A, B). When the glass rod was presented to the fish, but with the vibrations off, we detected no eye vergence nor eye tracking when the fish swam by the side of the glass rod.

Scale drawings showed that, just as in surface fish, freely swimming larvae have eyes pointing sub-perpendicular to their body, with the region of binocular overlap (blue) being minimal (Fig. 5C). During LPC, mean vergence angle also changes in the cavefish, advancing the binocular field to close to the front of the mid-point of the eyes (Fig. 5D). In the case of the cavefish larvae used in this experiment, changes in the binocular field serve little function as visual space may be irrelevant due to the degree of blindness by this stage.

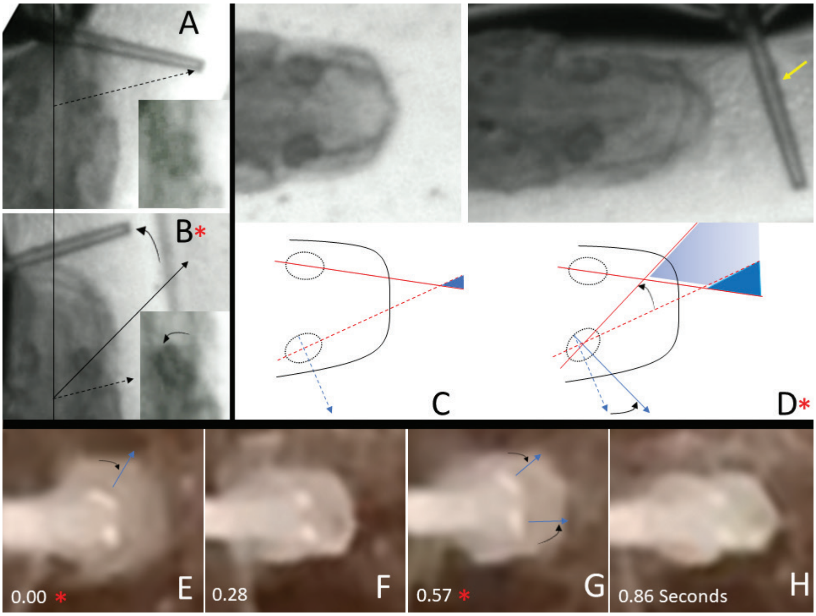

Figure 5. Despite being blind, the eyes of cavefish larvae tracked the position of the source of vibrations (**A, B**). During LPC, the mean vergence angle changes in the cavefish, advancing the binocular field to close to the front of the mid-point of the eyes (**C, D**). Each eye can have its own and different angle of vergence (**E–H**). Depending on the position of the source of the vibrating stimulus, a single eye may move forward, while the other remains laterally pointing (**E**). At another position of the stimulus, both eyes may converge forward (**G**). Soon after, eyes return to normal position (**F, H**). Eye vergence may represent a vestigial behavioral character, left-over in the evolution of *Astyanax* cavefish, since changes in the binocular field may be irrelevant due to the blindness of larvae by this stage of development and because of living in the dark with no visual stimuli.

Each eye can have its own vergence. Depending on the position of the source of the vibrating stimulus, a single eye may move forward while the other remains laterally pointing (Fig. 5C, D and 5E, F). At another position, both eyes may converge forward (Fig. 5G, H). In blind cavefish *Astyanax* larvae, it appears that non-visual stimuli activate LPC concurrently with ocular convergence. Direct contact with the body by the source of vibrations is unnecessary, and vibrations at a frequency of ~10 Hz activate the ocular vergence at a distance.

Discussion

Astyanax cavefish have been reported to be blind and lack physiological response to light in the tectum (Voneida and Fish 1984), but Lloyd et al. (2022) found that the cavefish tectum showed some, although severely reduced, Ca2+ responses to the presented visual stimuli. Nonetheless, at least in the case of the Pachón population, the retina does not respond to light and the light responsiveness observed in the optic tectum of cavefish must stem from a non-visual source such as the pineal gland or deep-brain internal photoreceptors (Lloyd et al. 2022).

Our behavioral data suggest that surface *Astyanax* fish may have several prey-capture specific motor programs. One may start with a visual stimulus that activates ocular convergence for enhanced binocular processing of visual information. This is followed by the appropriate J-turns of the tail, or C-bend turns movement for a strike towards the prey. Another motor program may start with non-visual stimuli, such as a 10 Hz vibration. This may activate in synchrony the J- or C- turns while positioning the eyes in convergence. The advantage of this synchronous activation is that under light conditions when the fish has positioned itself for the final strike motion based on vibration information, binocular optimization can then occur. This is supported by observations in zebrafish (Bianco et al. 2011); eye convergence during hunting behavior in larval zebrafish is not to direct gaze toward the prey, because the eyes adopted a symmetrical converged configuration independent of prey location. Rather, it is more likely that eye convergence acts to increase the binocular overlap between the visual fields of the left and right eyes in preparation for prey tracking. If visual information does not occur such as at night, in *Astyanax* surface fish, the strike is activated only with the available data, and eye vergence becomes irrelevant. This is also supported in zebrafish (Patterson et al. 2013), where while in the dark, paramecia trigger ocular vergence. A difference between zebrafish and *Astyanax*, both surface and cave, is that while in zebrafish the strike is activated only when the prey enters in contact with the anterior extremities of fish larvae, in *Astyanax* prey is detected at a distance (Espinasa et al. in press). *Astyanax* may rely more on other non-visual sensory modes that result in more efficient prey hunting in the dark than Zebrafish. *Astyanax* cavefish can strike at prey at farther distances than *Astyanax* surface fish (Lloyd et al. 2018: Espinasa et al. in press). The increased distance of the strike may contribute to the enhanced feeding of cavefish, as seen in a competition assay in 17 dpf fry, where cavefish consumed more *Artemia* than surface fish under dark conditions (Espinasa et al. 2014).

For cavefish, the second motor program may be at work. LPC may start with a non-visual stimulus, such as a 10 Hz vibration, smell, or sound. This may activate in synchrony the C- or J- turns while positioning the eyes in convergence. In the case of the blind cavefish, the vergence of the eyes may currently serve no function while in the dark environment of the cave and be a left-over of evolution, where natural selection or other evolutionary forces have not regressed this behavior. The activity of premotor neurons producing eye convergence commands is assumed to have been a fundamental component of the activity pattern underlying all behavioral responses to prey-like stimuli in the ancestral surface fish that gave rise to the cavefish. Just as it may be in the existing surface *Astyanax* fish. This activity has not regressed at the same pace as structural eye degeneration.

Espinasa and Jeffery (2006) showed that loss of physiological function does not necessarily precede or occur at the same pace as structural degeneration during regressive evolution in the cavefish eye. They showed that the capacity to exhibit light-entrained retinomotor rhythms has been conserved in the degenerating embryonic eyes of *Astyanax* cavefish populations. The results indicate that loss of circadian retinal function does not precede and is, therefore, not required for eye degeneration in blind cavefish. In *Astyanax* surface fish during the day, the retinal pigment epithelium (RPE) extends to shield the rod photoreceptor outer segments, reducing the extent of bleaching. During the night, it retracts to expose the photoreceptors, allowing them to catch the maximum number of photons. These retinomotor rhythms would appear to have no function in blind cavefish. Therefore, movements of unpigmented RPE granules (e.g., Pachón cavefish) would not be needed to reduce the extent of bleaching of photoreceptors, which are absent in cavefish. Likewise, maintenance of retinomotor activity would not be expected to optimize visual capabilities in cavefish, which do not respond to visual stimuli in the laboratory (Voneida and Sligar 1976; Voneida and Fish 1984). Therefore, they considered retinomotor rhythms a vestigial physiological character in *Astyanax* cavefish.

Retinomotor rhythms and ocular vergence may have been preserved fortuitously in the degenerating cavefish eye evolution. The persistence of the retinomotor movements in response to a circadian rhythm, and the eye vergence during LPC, suggest that both are controlled by genetic and physiologic signals independent of degenerating cavefish eye. The expression of sonic hedgehog and tiggy-winkle hedgehog genes is enhanced along the anterior midline of cavefish embryos (Yamamoto et al. 2004). Consequently, the increase in hedgehog signaling causes eye degeneration by triggering lens apoptosis. The signals that regulate eye degeneration are independent and nonconflicting with those that control retinomotor movements and eye vergence.

Conclusions

The blind tetra, *Astyanax mexicanus*, despite being adapted to the absence of visual stimuli due to the lack of light in the cave, have retained the oculomotor behavior of eye convergence in their vestigial eyes as a response to prey stimuli. In *Astyanax*, eye convergence responses can be triggered singlehandedly by vibrations elicited with a glass rod at frequencies similar to those generated by its prey (10–35 Hz).

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RESEARCH ARTICLE

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New records of cave-dwelling populations of *Rhamdia* catfishes (Siluriformes, Heptapteridae) from Chiapas, Mexico

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Abstract

Dedicated ichthyological surveys in four active karstic caves in the Mexican state of Chiapas (Grijalva River drainage basin) resulted in the discovery of the same number of hypogean populations of *Rhamdia* catfishes assignable to two different species: *R. laticauda* and *R. guatemalensis*. The taxonomic identity of these populations was initially determined based on morphological traits and subsequently corroborated with molecular data in a phylogenetic framework. For the most part, these newly discovered populations exhibit partial and variable troglomorphism (vs. fixed), a pattern that has been observed in most other cave-dwelling species/populations of Mexican *Rhamdia*, and possibly caused by gene flow with and/or incipient speciation from epigean lineages. Since most hypogean forms of Mexican *Rhamdia* derive from/are part of a larger *R. laticauda* clade, our discovery of cave-dwelling populations assignable to *R. guatemalensis* is noteworthy and includes the very first record of a *R. guatemalensis* population with pronounced and widespread troglomorphism. Our discovery of hitherto unrecorded populations of hypogean *Rhamdia* highlights the continued importance of exploration in the process of documenting subterranean biodiversity, particularly in regions of the world rich with cave systems. Our findings corroborate the notion that, among Neotropical fishes, the catfish genus *Rhamdia* is one of the most prone and effective at colonizing subterranean habitats and establishing viable hypogean populations.

Keywords
Cavefishes, hypogean populations, troglobitic fishes, troglomorphism

Introduction

Catfishes of the genus *Rhamdia* Bleeker, 1858 are a clade of Neotropical freshwater fishes widely distributed throughout the continent–from Mexico to Argentina–and with considerable taxonomic diversity (27 currently valid species) (Silfvergrip 1996; Perdices et al. 2002; Hernández et al. 2015; Angrizani and Malabarba 2020; Fricke et al. 2020). Remarkably, *Rhamdia* catfishes have a propensity to colonize subterranean habitats, particularly in the form of active karst caves, as evidenced by the fact that about a quarter of their overall species diversity, plus several populations lacking definite species adscription, are stygobitic (living exclusively in groundwater) (Arroyave and De La Cruz Fernández 2021a, b).

The six valid species of hypogean (cave-dwelling) and troglobitic (displaying phenotypic adaptations to cave life) *Rhamdia* are: the Brazilian *Rhamdia enfurnada* (Bichuette and Trajano 2005), the Venezuelan *Rhamdia guasarensis* (DoNascimiento et al. 2004), and the Mexican *Rhamdia reddelli* (Miller 1984), *Rhamdia zongolicensis* (Wilkens 1993), *Rhamdia macuspanensis* (Weber and Wilkens 1998), and *Rhamdia laluchensis* (Weber et al. 2003). Notably, all of them are characterized by being fully troglomorphic and microendemic to their respective type-locality caves, at least based on their original descriptions. Meanwhile, cave-dwelling *Rhamdia* populations of dubious taxonomic designation and with varying degree of troglomorphism have so far only been reported for Mexico (Robertson 1983; Mosier 1984; Arroyave and De La Cruz Fernández 2021a, b).

As evidenced by the above, the bulk of the known diversity of hypogean *Rhamdia* resides in Mexico, which is rather unsurprising, given that roughly 20% of Mexican territory is karstic landscape (Bautista 2023). In Mexico, *Rhamdia* catfishes have colonized karst formations in the southeast, with most populations (at least five taxonomically unassigned, although probably *R. laticauda*) and species (*R. zongolicensis* and *R. reddelli*) reported from the mountainous karst region of the Sierra de Zongolica, state of Veracruz, bordering with the state of Oaxaca, Papaloapan River basin (Arroyave and De La Cruz Fernández 2021a, b). Only after ~350 km southeast of Zongolica do other cave *Rhamdia* species occur, in the state of Chiapas (*R. laluchensis*) and further east in Tabasco (*R. macuspanensis*), in active caves that are part of the Grijalva-Usumascinta River basin (Arroyave and De La Cruz Fernández 2021b).

Besides cave-dwelling forms, Mexican *Rhamdia* include the surface species *Rhamdia laticauda*, *Rhamdia guatemalensis*, and *Rhamdia parryi* (Miller 2005). Existing research on the evolutionary history of *Rhamdia* in Middle America suggests that the diversity of cave-dwelling lineages in the region derives from the epigean and more widespread species *R. laticauda*, with the possibility that all Mexican cave-dwelling forms (including all four valid species) are in fact cave-adapted populations of *R. laticauda* (Perdices et al. 2002; Arroyave and De La Cruz Fernández 2021a, b). To date, the only known exception to this pattern (*R. laticauda* being the "ancestor" of all cave *Rhamdia* in Mexico) would be a non-troglomorphic cave-dwelling population of *Rhamdia* from the Grutas de Coconá (Tabasco), assignable to the surface species *R. guatemalensis* (Arroyave and De La Cruz Fernández 2021b).

While a renewed interest and recent research including field surveys and exploration has resulted in improved understanding of the taxonomic diversity and evolutionary history of Mexican cave *Rhamdia* (Arroyave and De La Cruz Fernández 2021a, b), vast extensions of karstic landscape remain to be surveyed for the presence of hypogean populations. The state of Chiapas, with its rich karstic topography that includes the Sierra Madre de Chiapas, the Chiapas Highlands, and the Chiapas Depression, offers a promising target for the discovery and documentation of previously unknown and/or understudied hypogean populations of *Rhamdia*. Besides the formally described troglobitic species *Rhamdia laluchensis* (Weber et al. 2003), existing records of hypogean catfishes in caves of Chiapas are limited to a few reports from historical international speleological expeditions in the region and from anecdotical accounts from local spelunkers, namely: Sistema Pecho Blanco (Cintalapa de Figueroa) (Sbordoni et al. 1986; Sbordoni and Lucarelli 1989), Cueva de los dos Hermanos (Berriozabal) (Sbordoni et al. 2004), an unnamed cave in the Selva del Mercadito (Sbordoni and Lucarelli 1989), El Chorro Grande cave (Río Suchiapa) (http://www.oztotl.com/ps/reports/El%20Chorro%20Grande.pdf, http://eksa.free.fr/chiapas2008/chiapas2008.pdf), Paso Burro cave (Berriozabal), and Los Bordos and El Encanto caves (Río La Venta). None of these gray-literature and anecdotical reports, however, has been previously investigated and documented from a taxonomic standpoint. Consequently, we embarked on an ichthyological expedition devoted to survey and sample some of these caves. This study presents our findings, documenting hitherto unknown cave-dwelling populations of *Rhamdia* catfishes from the state of Chiapas, and shedding light on their taxonomic nature and evolutionary history based on analyses of morphological and genetic comparative data.

Methods

Area of study

From March 17th to 25th, 2022, we conducted ichthyological surveys in four active, resurgence, semidry (vs. submerged), and relatively horizontal karstic caves located in the Mexican state of Chiapas, within the Grijalva River drainage basin, including three of its tributaries. Two of the surveyed caves, Los Bordos (16°49'48.70"N, 93°31'33.70"W) and El Encanto (16°45'26.8"N, 93°31'30.6"W), are part of the Río La Venta basin, while the other two caves, Paso Burro (16°49'53.3"N, 93°16'29.2"W) and El Chorro Grande (16°31'13.0"N, 93°14'39.0"W), are part of the Río Sabinal and Río Suchiapa basins, respectively (Figs 1, 2). Los Bordos, a relatively high discharge cave containing rather deep ponds (~2 m), develops a total of 5211 m in length and has a vertical gradient of +58 m from the entrance (Barbe and Morenas 1989). El Encanto is a much shorter cave (364 m) and with a much smaller vertical gradient (-4, +9 m) (Whitaker 1988; Barbe and Morenas 1989). The resurgence of El Chorro Grande opens at the bottom of the Río Suchiapa canyon, and, in the rainy season, the water gushes violently from the porch, hence its name (The Great Jet). El Chorro Grande cave develops 9650 m and has a vertical gradient of +175 m throughout its entire length (http://eksa.free.fr/chiapas2002/chiapas2002.pdf). The resurgence of Paso Burro feeds the Sabinal River and provides water for the municipality of Berriozabal (Whitaker 1988). Los Bordos and El Chorro Grande are relatively remote, hard-to-reach, non-touristy caves that require considerable hiking time and effort (mostly through the Río La Venta riverbed) followed by a rather steep ascent through the canyon wall up to the cave entrance. Conversely, El Encanto (also known as Cueva del Aguacero) is a touristy cave easily accessible by motor vehicle and therefore barely requiring any hiking. In between these extremes is Paso Burro cave, which only requires a relatively short and effortless hike from the point of closest car access.

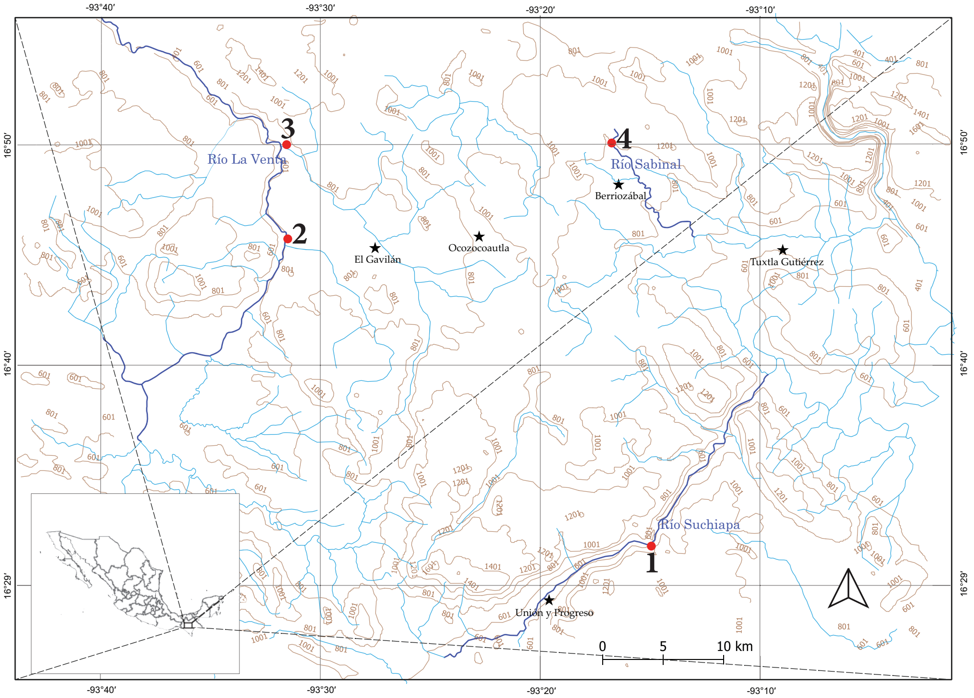

Figure 1. Map of the area of study showing the location of the surveyed caves with hitherto undocumented populations of hypogean *Rhamdia*. Cave localities are indicated by red dots and correspond to: **1** El Chorro Grande cave (Río Suchiapa basin) **2** El Encanto cave (Río La Venta basin) **3** Los Bordos cave (Río La Venta basin), and **4** Paso Burro cave (Río Sabinal basin).

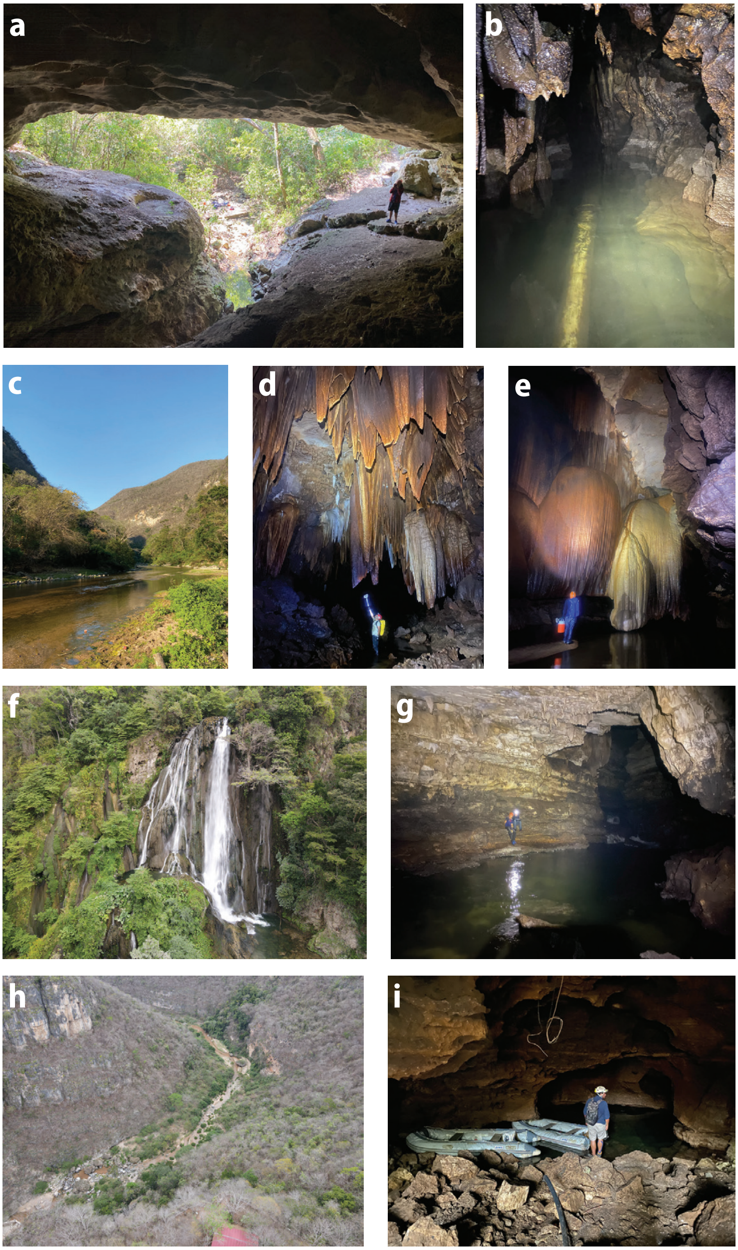

Figure 2. Images of hypogean and epigean localities surveyed in this study **a** main entrance of Paso Burro cave **b** interior of Paso Burro cave where it can be seen an underwater aqueduct pipe traversing the main channel **c** Río Suchiapa riverbed near the access point to El Chorro Grande cave **d, e** Inside of El Chorro Grande cave, showing its massive cross-section size and different types of correspondingly enormous speleothems **f** aerial view of La Conchuda waterfall, which flows into the Río La Venta from the Los Bordos cave **g** interior of the Los Bordos cave showing a pool where fishes where collected **h** aerial view of the Río La Venta canyon at El Aguacero waterfall, near El Encanto cave (located in the same premises as the red roof house seen at the bottom of the photograph **i** inside El Encanto cave at entrance point, showing boats normally used by tourists who want to navigate the interior of the cave.

Specimen sampling and preservation

We collected cave-dwelling *Rhamdia* specimens using baited minnow traps deployed along the main longitudinal axis of the cave, up until our point of maximum penetration (approximately a few hundred meters in all four caves). For comparative purposes, in addition to hypogean populations, we sampled epigean *Rhamdia* by means of electrofishing at river locations in the vicinity of the sampled caves. We were able to sample epigean populations nearby all surveyed caves except El Chorro Grande (Río Suchiapa basin). After capture, we euthanized the fishes using the anesthetic tricaine mesylate (MS-222) and then took tissue samples (fin clips) for genetic studies. Tissues were preserved in in 96% ethanol and eventually cryopreserved at −80 °C. After tissuing, we fixed voucher specimens using a 10% formalin solution. Back in the lab, we washed formalin-fixed specimens and then transferred them to 70% ethanol for long-term storage in the Colección Nacional de Peces (CNPE) of the Instituto de Biología, Universidad Nacional Autónoma de México (IBUNAM), where they have been catalogued and deposited (Table 1). Fishes were handled in accordance with recommended guidelines for the use of fishes in research (Jenkins et al. 2014). Specimens were collected under permit SGPA/DGVS/08073/21 issued by the Mexican Ministry of Environment and Natural Resources (Secretaría de Medio Ambiente y Recursos Naturales; SEMARNAT).

Comparative data generation and analysis

To document patterns of phenotypic and genetic variation, and to shed light on the taxonomic nature of these newly discovered cave-dwelling *Rhamdia* populations, we collected morphometric and meristic data from all hypogean specimens sampled, as well as DNA sequence data from a reduced subset. Traditional morphometric measurements and meristic counts follow previous taxonomic studies of *Rhamdia* (Silfvergrip 1996; Hernández et al. 2015). All measurements were taken on the left side of the specimen using a Mitutoyo digital caliper (precision = 0.1 mm; accuracy = ±0.02 mm). To generate comparative genetic data, we first extracted total genomic DNA from fresh tissue samples using the Qiagen DNeasy Tissue Extraction Kit, following the manufacturer's protocol. Subsequently, we amplified and sequenced a partial fragment (~650 bp) of the mitochondrial marker cytochrome c oxidase subunit I (COI) using the primer pairs LCO1490/HCO2198 (Folmer et al. 1994). DNA extraction, amplification, and sequencing were carried out at Laboratorio de Secuenciación Genómica de la Biodiversidad y de la Salud (Instituto de Biología, UNAM), in-house Sanger sequencing facilities. Contig assemblage, sequence editing, and multiple sequence alignment were accomplished using Geneious Prime 2023.0.2 (https://www.geneious.com). We sequenced a total of 18 individuals: eight representing all newfound hypogean populations and 10 from epigean populations immediately adjacent to three of the four caves surveyed (Los Bordos, El Encanto, and Paso Burro) (Table 1). To broaden the taxonomic and geographic coverage of samples used for phylogenetic analysis, we also mined additional *Rhamdia* COI sequences from GenBank (www.ncbi.nlm.nih.gov/Genbank) and BOLD (www.barcodinglife.org) (37) and from unpublished data (26) previously generated by the senior au-

Table 1. *Rhamdia* specimens collected during the fieldwork component of this study, including samples from four newly discovered hypogean populations and from four epigean populations in the vicinity of three of the surveyed caves, with their respective catalog and voucher numbers. GenBank accession numbers correspond to COI sequenced data generated herein and used for phylogenetic analysis.

Habitat	Locality	Coordinates	Basin	Species	Catalog	Voucher	COI GenBank accession
Hypogean	Los Bordos cave	16°49'48.70"N, 93°31'33.70"W	La Venta	*Rhamdia guatemalensis*	CNPE-IBUNAM 24020	JA1294	OR512373
					CNPE-IBUNAM 24020	JA1295	OR512374
					CNPE-IBUNAM 24020	JA1296	OR512375
					CNPE-IBUNAM 24020	JA1297	n/a
					CNPE-IBUNAM 24020	JA1298	n/a
					CNPE-IBUNAM 24020	JA1299	n/a
					CNPE-IBUNAM 24020	JA1300	n/a
					CNPE-IBUNAM 24020	JA1301	n/a
	El Encanto cave	16°45'26.8"N, 93°31'30.6"W	La Venta	*Rhamdia guatemalensis*	CNPE-IBUNAM 24021	JA1412	OR512387
	Paso Burro cave	16°49'53.3"N, 93°16'29.2"W	Sabinal	*Rhamdia laticauda*	CNPE-IBUNAM 24022	JA1272	OR512370
					CNPE-IBUNAM 24022	JA1273	OR512371
					CNPE-IBUNAM 24022	JA1309	OR512379
					CNPE-IBUNAM 24022	JA1310	n/a
					CNPE-IBUNAM 24022	JA1311	n/a
					CNPE-IBUNAM 24022	JA1312	n/a
					CNPE-IBUNAM 24022	JA1313	n/a
	El Chorro Grande cave	16°31'13.0"N, 93°14'39.0"W	Suchiapa	*Rhamdia laticauda*	CNPE-IBUNAM 24023	JA1275	n/a
					CNPE-IBUNAM 24023	JA1276	OR512372
					CNPE-IBUNAM 24023	JA1277	n/a
					CNPE-IBUNAM 24023	JA1278	n/a
					CNPE-IBUNAM 24023	JA1279	n/a
					CNPE-IBUNAM 24023	JA1280	n/a
					CNPE-IBUNAM 24023	JLP3R3	n/a
					CNPE-IBUNAM 24023	JLP1R1	n/a
Epigean	Río La Venta at La Conchuda waterfall	16°49'57.42"N, 93°31'52.3"W	La Venta	*Rhamdia guatemalensis*	CNPE-IBUNAM 24024	JA1302	OR512376
					CNPE-IBUNAM 24024	JA1303	OR512377
					CNPE-IBUNAM 24024	JA1304	OR512378
	Río La Venta at El Aguacero waterfall	16°45'40.16"N, 93°31'33.1"W	La Venta	*Rhamdia guatemalensis*	CNPE-IBUNAM 24027	JA1359	OR512384
					CNPE-IBUNAM 24027	JA1360	OR512385
					CNPE-IBUNAM 24027	JA1361	OR512386
	Río Sabinal at Paso Burro cave outflow	16°49'33.3"N, 93°16'13.7"W	Sabinal	*Rhamdia guatemalensis*	CNPE-IBUNAM 24025	JA1314	OR512380
					CNPE-IBUNAM 24025	JA1315	OR512381
					CNPE-IBUNAM 24025	JA1316	OR512382
				Rhamdia laticauda	CNPE-IBUNAM 24026	JA1320	OR512383

thor (JA). Ultimately, we assembled a molecular data matrix that included most species (90%) of the trans-Andean/Middle American *Rhamdia* clade, totaling 81 terminals and 640 aligned positions. We inferred a phylogenetic tree based on the resulting COI matrix (with *Rhamdia quelen* as outgroup) using the software RAxML-NG (v. 1.0.1) (Kozlov et al. 2019) under the HKY+I+G model of molecular evolution. Statistical selection of the best-fit model of nucleotide substitution was implemented with the software jModelTest2 (v. 2.1.10) (Darriba et al. 2012) under the following likelihood settings: number of substitution schemes = 3; base frequencies = +F; rate variation = +I and +G with nCat = 4; base tree for likelihood calculations = ML optimized; and base tree search = NNI, effectively evaluating 24 models. Clade support was estimated using the bootstrap character resampling method (Felsenstein 1985) based on 1000 pseudoreplicates.

Results

Our ichthyological surveys in four cave systems in the state of Chiapas, Mexico, resulted in the discovery of four hitherto undocumented hypogean populations of catfishes of the genus *Rhamdia*, three of which with individuals only partially (vs. completely) troglomorphic, and to a varying degree (Fig. 3). Despite its cave-dwelling nature, the only specimen collected at El Encanto cave displayed normal (non-troglomorphic) phenotype (Fig. 3). Specimens from these four newly discovered populations were initially ascribed, based on external morphology, to the epigean species *R. guatemalensis* (Los Bordos and El Encanto caves) and *R. laticauda* (Paso Burro and El Chorro Grande caves), a result that was further corroborated by comparative molecular data in a phylogenetic context (Fig. 4). Morphological and meristic data from the specimens collected at the surveyed caves are presented in Tables 2, 3. The phylogeny presented in Fig. 4 clearly shows that

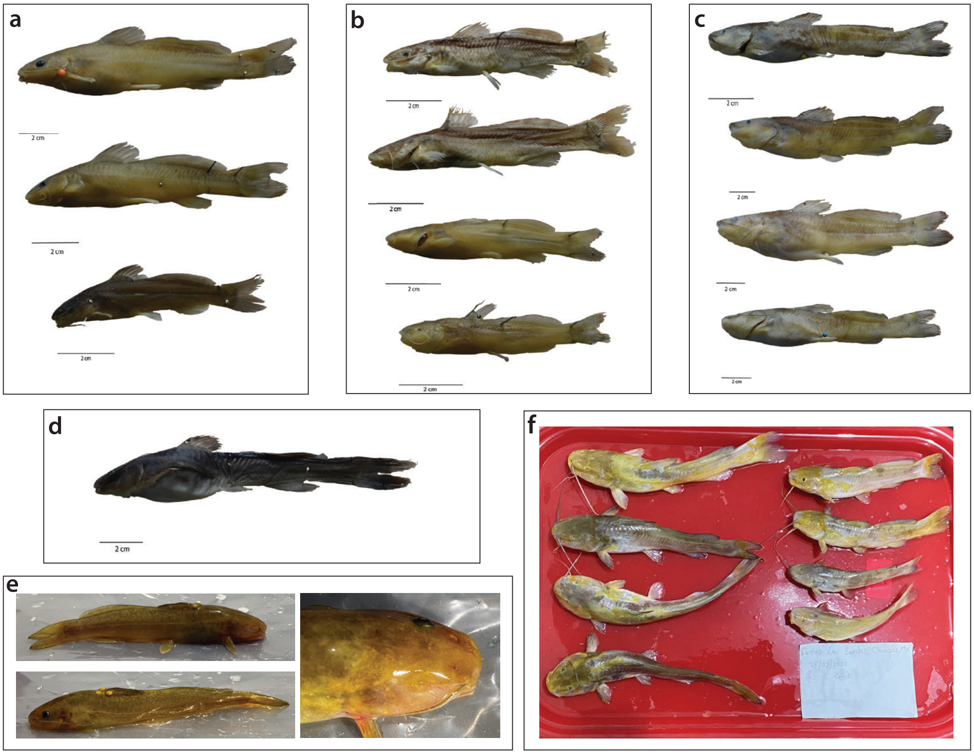

Figure 3. Specimens of newly discovered populations of hypogean *Rhamdia* from Chiapas, showing observable external morphological features, including variation in the degree of troglomorphism (eye reduction and depigmentation) **a** preserved specimens of *R. laticauda* from Paso Burro cave **b** preserved specimens of *R. laticauda* from El Chorro Grande cave **c** preserved specimens of *R. guatemalensis* from Los Bordos cave **d** preserved specimen of *R. guatemalensis* from El Encanto cave **e** fresh specimen (prior to euthanasia and formalin fixation of *R. laticauda* from Paso Burro cave in lateral (body, both sides) and dorsal (head) views, displaying partial troglomorphism characterized by depigmentation and complete absence of the right eye **f** fresh specimens of *R. guatemalensis* from Los Bordos cave, displaying a wide spectrum of variation in the degree of troglomorphism, including complete loss of eyes and pigmentation.

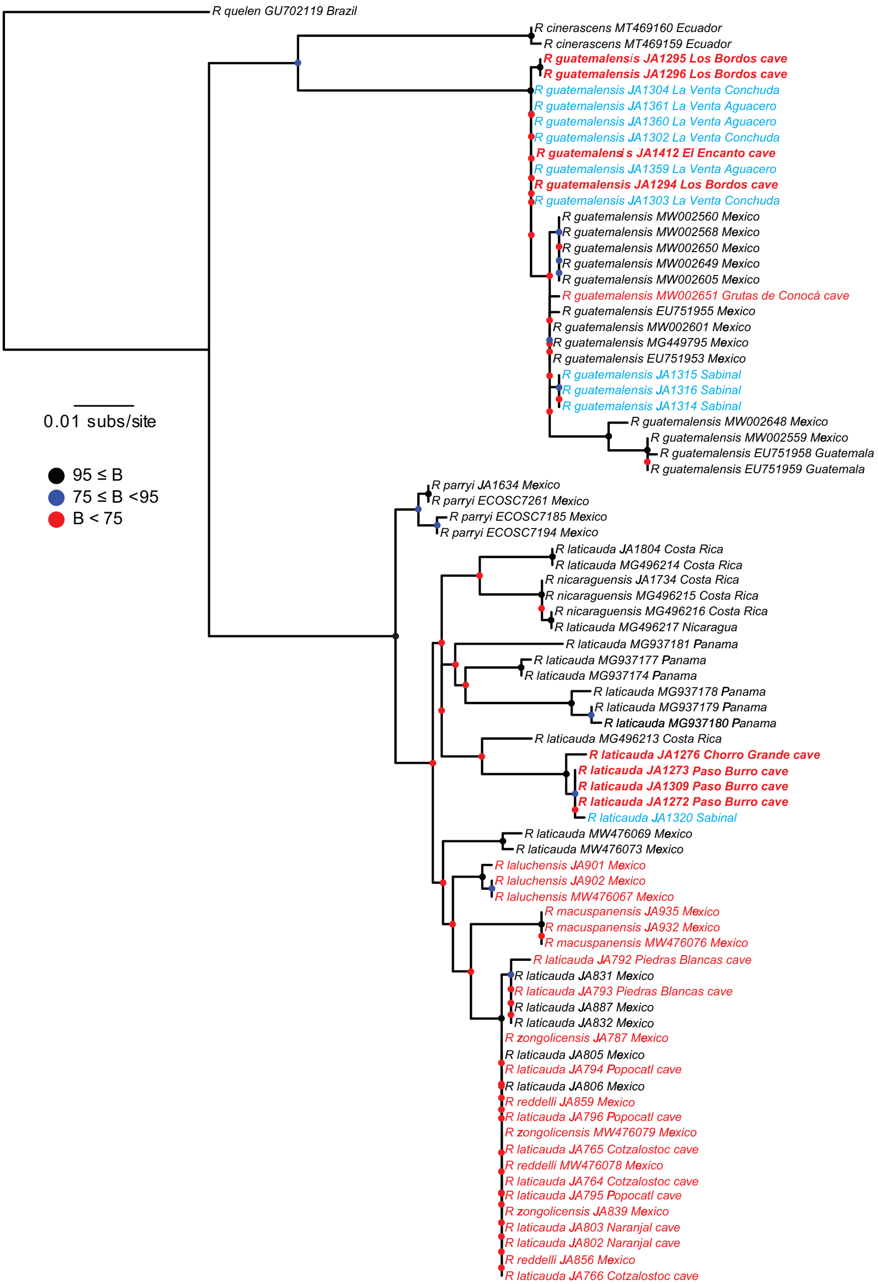

Figure 4. Phylogenetic tree of Middle American *Rhamdia* based on comparative COI sequence data, highlighting the phylogenetic position of individuals from the newly discovered hypogean populations as well as from epigean populations near the surveyed caves. Cave-dwelling species/populations in red (newfound in bold). Epigean species/populations in black, except for localities surveyed herein, in blue. Terminals corresponding to COI sequences obtained from GenBank (www.ncbi.nlm.nih.gov/Genbank), BOLD (www.barcodinglife.org), or from unpublished data previously generated by the senior author (JA), include the corresponding accession or voucher/catalog number in the label (between the species name and the country of origin). Colored circles on nodes indicate degree of clade support as determined by bootstrap values (B).

the newly discovered cave-dwelling populations of *Rhamdia* documented herein (colored in bold red) are well nested within two major species-level clades that correspond to the epigean *R. guatemalensis* (Los Bordos and El Encanto caves) and *R. laticauda* (Paso Burro and El Chorro Grande caves), respectively. The phylogeny also shows that samples from Los Bordos and El Encanto are closely associated with and almost genetically indistinguishable from epigean samples from the Río La Venta (colored in blue) collected at El Aguacero and La Conchuda waterfalls. Within the *R. guatemalensis* clade, the only other known hypogean population is found in the Grutas de Coconá cave system, in the state of Tabasco. As shown in the phylogeny, the bulk of the diversity of hypogean Middle American *Rhamdia* is however represented by populations of *R. laticauda*, including two documented for the first time in this study: El Chorro Grande and Paso Burro caves. Unsurprisingly, the most closely related sample to those from Paso Burro cave is from an epigean population found in the river flowing out of the cave (Río Sabinal). While there is strong nodal support for the clade consisting of El Chorro Grande, Paso Burro, and Sabinal samples, its relationship to other components of the *R. laticauda* radiation is rather uncertain (due to the low support of the nodes involved). Despite this fact, it appears that this clade is more closely related to *R. laticauda* Central American lineages (including *R. nicaraguensis*) than to those occurring further north in Mexico, including cave-dwelling species and populations found in Veracruz (*R. zongolicensis* and hypogean *R. laticauda*), Oaxaca (*R. reddelli*), Chiapas (*R. laluchensis*), and Tabasco (*R. macuspanensis*).

Table 2. Meristic comparative data from specimens from the newly discovered hypogean populations. Meristic traits abbreviations as follows: PFR = pectoral-fin rays, PvFR = pelvic-fin rays, DFR = dorsal-fin rays, ARF = anal-fin rays, uCFR = upper caudal-fin rays, and lCFR = lower caudal-fin rays. Caudal-fin rays numbers (x,y,z) correspond to unsegmented (x), unbranched segmented (y), and branched segmented (z) rays.

Cave	Species	Voucher	PFR	PvFR	DFR	AFR	uCFR	lCFR
Paso Burro	*Rhamdia laticauda*	JA1272	I-7	6	I-7	8	4,2,6	4,2,5
		JA1273	I-8	6	I-6	7	4,2,6	4,2,5
		JA1309	I-9	6	I-7	9	5,2,6	4,2,5
		JA1310	I-10	6	I-7	10	5,2,6	4,2,5
		JA1311	I-10	6	I-7	10	4,2,6	4,2,5
		JA1312	I-9	6	I-7	10	4,2,6	4,2,5
		JA1313	I-9	5	I-7	10	4,2,6	4,2,5
El Chorro Grande	*Rhamdia laticauda*	JA1275	I-8	5	I-5	8	4,2,6	4,2,4
		JA1276	I-8	6	I-7	9	4,2,6	4,2,5
		JA1277	I-7	5	I-6	8	4,2,6	4,2,5
		JA1278	I-7	6	I-6	9	4,2,6	4,2,4
		JA1279	I-8	6	I-6	9	4,2,6	4,2,5
		JA1280	I-8	5	I-6	7	4,2,6	4,2,5
		JLP1R1	I-8	7	I-6	10	4,2,6	4,2,5
		JLP3L3	I-6	6	I-6	10	4,2,6	4,2,5
Los Bordos	*Rhamdia guatemalensis*	JA1294	I-9	6	I-6	10	4,2,6	4,2,6
		JA1295	I-7	6	I-6	10	4,2,5	3,2,5
		JA1296	I-7	6	I-6	11	4,2,6	4,2,5
		JA1297	I-7	6	I-6	10	4,2,6	4,2,6
		JA1298	I-7	6	I-6	10	4,2,6	4,2,6
		JA1299	I-7	6	I-6	10	4,2,6	4,2,6
		JA1300	I-7	6	I-6	11	5,2,6	4,2,5
		JA1301	I-7	6	I-6	10	4,2,6	4,2,6
El Encanto	*Rhamdia guatemalensis*	JA1412	I-9	6	I-7	6	7,2,5	6,2,4

Table 3. Morphometric comparative data from specimens from the newly discovered hypogean populations. Measurements abbreviations as follows: SL = Standard Length, HL = Head Length, HL = Head Length, BW = Body Width, DFH = Dorsal Fin Height, DSH = Dorsal-fin Spine Height, AFL = Anal Fin Length, AdFL = Adipose Fin Length, PFL = Pectoral Fin Length, PSL = Pectoral-fin Spine Length, PvFL = Pelvic Fin Length, ISL = Interdorsal Space Length, CPL = Caudal Peduncle Length, CPD = Caudal Peduncle Depth, IOW = Interorbital Width, ORB = Orbital Diameter, SNT = Snout Length, MBL = Maxillary Barbel Length, MdBL = Mandibular Barbel Length, MeBL = Mental Barbel Length.

	Rhamdia laticauda						***Rhamdia guatemalensis***			
	Paso Burro (N = 7)			**El Chorro Grande (N = 8)**			**Los Bordos (N = 8)**			**El Encanto (N = 1)**
	Range	**Mean**	**SD**	**Range**	**Mean**	**SD**	**Range**	**Mean**	**SD**	**Value**
mm										
SL	57.3–122.2	88.07	23.15	48.8–89.9	67.41	14.18	116.6–183.2	150.26	25.45	125.70
HL	13.2–22.2	17.56	3.48	11.1–17.7	13.80	2.38	25.6–42.7	33.93	6.89	23.70
% SL										
HL	17.87–23.73	20.30	2.12	18.41–21.17	20.16	1.04	20.76–24.52	22.47	1.36	18.85
BW	17.04–18.32	17.72	0.40	17.38–18.80	18.16	0.45	17.84–19.94	18.79	0.75	17.42
DFH	9.72–15.36	12.10	1.82	7.30–13.25	10.49	2.29	9.32–11.90	10.47	0.99	12.89
DSH	2.79–6.34	4.84	1.26	4.04–7.34	6.02	0.99	6.25–8.83	7.79	0.84	9.31
AFL	4.03–6.26	5.22	0.86	3.51–7.22	4.86	1.38	4.29–6.90	5.83	0.94	3.26
AdFL	31.73–36.36	34.39	1.59	22.02–32.22	26.51	3.35	27.77–34.29	31.36	2.19	43.91
PFL	10.61–15.01	12.23	1.36	12.53–14.76	13.38	0.85	11.88–13.88	12.82	0.66	13.92
PSL	3.26–5.76	4.49	0.79	5.62–9.45	6.84	1.30	6.65–10.26	9.09	1.26	8.99
PvFL	11.64–13.78	12.47	0.89	10.43–12.39	11.76	0.57	10.15–13.29	11.30	1.03	12.81
ISL	8.59–9.95	9.47	0.48	9.30–17.79	14.49	2.68	5.95–9.69	7.12	1.32	1.75
CPL	20.19–23.21	21.75	1.20	17.40–23.41	20.89	2.07	23.04–24.64	23.88	0.51	24.26
CPD	9.78–10.41	10.07	0.23	7.73–10.90	9.62	1.07	9.52–11.89	10.60	0.91	7.16
% HL										
IOW	31.61–38.86	36.45	2.80	36.44–47.75	41.95	3.82	33.59–44.00	37.84	3.54	51.90
ORB	14.07–20.00	17.67	2.24	2.50–11.41	7.68	3.49	5.86–13.96	9.34	2.66	18.14
SNT	34.37–43.43	39.45	3.31	39.84–47.75	42.54	2.81	38.53–48.80	42.72	3.64	38.40
MBL	100–172	129.11	25.14	114.69–177.48	153.99	23.31	151.54–201.04	178.54	19.27	228.69
MdBL	60.57–81.82	69.54	6.93	38.46–86.18	68.41	15.75	65.48–98.62	78.88	11.68	91.56
MeBL	32.16–47.75	39.01	6.73	34.23–59.89	43.42	8.55	36.72–49.48	43.18	5.19	55.70

Discussion

The catfish genus *Rhamdia* is unarguably one of the most successful groups of freshwater fishes at colonizing hypogean habitats, particularly in Mexico, where most cave-dwelling species/populations are found, mainly in the Sierra de Zongolica, a karstic mountainous region that drains tributaries of the Papaloapan River basin in the state of Veracruz (Arroyave and De La Cruz Fernández 2021a). Prior to this study, the only taxonomically authoritative record of a cave-dwelling form of *Rhamdia* from the state of Chiapas corresponded to the species *R. laluchensis*, microendemic from the Sótano de La Lucha, a pit cave on the Grijalva River basin (Weber et al. 2003). The few grey-literature and anecdotical records of hypogean *Rhamdia* from Chiapas had neither been corroborated nor further investigated to establish the taxonomic nature of these populations. Our ichthyological surveys of four of these purportedly cavefish-harboring caves resulted in the rather unsurprising discovery of what appear to be well-established populations of hypogean *Rhamdia*, thus bringing the number of taxonomically verified (including catalogued vouchers) cave-dwelling populations of *Rhamdia* for the state of Chiapas up to five.

A first notable discovery from this study is that the *Rhamdia* specimens collected inside the surveyed caves do not represent undescribed species but populations of more widespread epigean species, specifically of *R. laticauda* and *R. guatemalensis*, and that each cave only harbors a single catfish species. Both morphological and molecular evidence strongly support this conclusion (Figs 3, 4). Whereas morphological identification of these populations was relatively straightforward (primarily based on pectoral spine serration pattern), characters traditionally used to distinguish between *R. laticauda* and *R. guatemalensis*, such as interdorsal space and adipose fin length, were not as clear-cut as expected (*R. laticauda* normally having a shorter adipose fin and correspondingly longer interdorsal space) (Table 3). Regardless of this potentially confounding morphological observation, phylogenetic analysis of comparative COI sequence data unequivocally resolved the populations from Paso Burro and El Chorro Grande as *R. laticauda* and those from Los Bordos and El Encanto as *R. guatemalensis* (Fig. 4). Furthermore, COI pairwise genetic distances between the newly discovered hypogean populations and their respective epigean species never exceeded the traditionally employed ~3% sequence divergence heuristic threshold for conspecifics (Hebert et al. 2003), providing additional support for the abovementioned species-level designation. Although our phylogenetic results also expose the non-monophyly of *R. laticauda* samples with respect to *R. nicaraguensis*, this pattern has been previously reported and discussed (Perdices et al. 2002; Arroyave and De La Cruz Fernández 2021b), and while deserving of further scrutiny, it is not the topic of our present study. This is, however, the subject of ongoing research by the senior author (JA) and collaborators, who are using genome-wide molecular markers and denser taxonomic and geographic sampling to address the systematics of the genus in Middle America.

The resulting phylogeny (Fig. 4) also makes absolute sense biogeographically, with samples from Paso Burro closely related–and almost genetically identical–to the epigean sample form Río Sabinal (river flowing out of the cave), and these samples altogether forming a clade sister to the sample from El Chorro Grande cave. Similarly, samples from Los Bordos and El Encanto caves were mainly resolved well nested within epigean samples from El Aguacero and La Conchuda waterfalls, an unsurprising result given that all four localities are part of the Río La Venta basin and in close proximity (no more than 10 km apart from each other). Furthermore, El Aguacero and La Conchuda waterfalls are located almost immediately outside of El Encanto and Los Bordos caves, respectively. Notably, the branch subtending the clade consisting of Paso Burro and El Chorro Grande samples (inclusive of the Río Sabinal sample) is particularly long, even longer than that of any of the troglobitic species currently recognized as distinct and valid (although nested within the *R. laticauda* radiation). While the causes behind such large genetic divergence are unclear at this point, this pattern certainly deserves attention and future scrutiny.

Another noteworthy finding of our study has to do with patterns of troglomorphism in cave *Rhamdia*. Except for El Encanto, for which no troglomorphic individuals were sampled (likely because of the small sample size, N = 1), all surveyed caves contained fishes with varying degree of troglomorphism (Fig. 3). This finding offers further support to the notion that hypogean fishes will eventually evolve a troglobitic phenotype characterized by eye reduction/loss and depigmentation, likely as adaptation to life in aphotic subterranean environments (Wilkens and Strecker 2017). Notably, complete troglomorphism (complete eye loss and depigmentation) is not fixed in any of the newly discovered hypogean populations. Instead, this condition is variable and only partial in most individuals sampled (Fig. 3). Partial troglomorphism in these populations is characterized by a varying degree of depigmentation and eye reduction (from normal to completely absent eyes). Remarkably, a specimen of hypogean *R. laticauda* from Paso Burro cave was found to display asymmetrical troglomorphism, lacking the right eye while still having a seemingly normal left one (Fig. 3e). The herein observed pattern of partial and variable troglomorphism conforms with recent research on Mexican cave-dwelling *Rhamdia* (Arroyave and De La Cruz Fernández 2021b), which uncovered that species believed to be completely troglomorphic, as per their original descriptions (Miller 1984; Wilkens 1993; Weber and Wilkens 1998; Weber et al. 2003), in reality display a spectrum of variation in the degree of troglomorphism. Such variable troglomorphism was also explicitly reported in the original description of the Brazilian *R. enfurnada* (Bichuette and Trajano 2005). Similarly, hypogean populations of *R. laticauda* from the Sierra de Zongolica have also been shown to exhibit clinical morphological variation in regressive troglomorphic traits (Arroyave and De La Cruz Fernández 2021a). Altogether, these findings suggest that varying (vs. fixed) troglomorphism might be the norm rather than the exception in hypogean *Rhamdia*. Although the exact causes behind this pattern have yet to be uncovered, time since cave colonization (incipient speciation) and crossings between hypogean and epigean individuals (gene flow) might be the main drivers constraining fixation of complete troglomorphism in these populations, a hypothesis recently advanced by previous authors (Arroyave and De La Cruz Fernández 2021b).

Admittedly, the discovery of hypogean populations of *R. guatemalensis* was contrary to our expectations, given that almost all known lineages of Mexican cave-dwelling *Rhamdia* derive from/are part of the *R. laticauda* radiation (Arroyave and De La Cruz Fernández 2021a, b). To our knowledge, the only prior record of a fully subterranean population of *R. guatemalensis* corresponds to the Grutas de Coconá cave system in the state of Tabasco (CNPE-IBUNAM 23815), with all three sampled specimens displaying a normal, non-troglomorphic phenotype (Arroyave and De La Cruz Fernández 2021b). As in the case of El Encanto cave, given this small sample size (N = 3), the existence of troglomorphic individuals in the population from Grutas de Coconá cannot be completely discounted. In any case, our discovery of two novel cave-dwelling populations of *R. guatemalensis* is noteworthy, particularly the one from Los Bordos cave, which effectively constitutes the very first record of a hypogean population of *R. guatemalensis* with pronounced and widespread troglomorphism (Fig. 3c, f). While some populations of *R. guatemalensis* from cenotes (karstic sinkholes) in the Yucatán Peninsula aquifer are considered somewhat hypogean, to the extent that they have been assigned subspecies status due to their incipient troglomorphism (i.e., *R. g. decolor* and *R. g. stygaea*) (Hubbs 1936), these populations are, strictly speaking, not troglobitic but trogloxenes, for the cenote is in most cases not a subterranean but an epigean habitat, and these fishes appear to only rarely venture into the cavern and cave zones of the aquifer (Arroyave et al. 2021).

We believe that the existence of hypogean fish populations, regardless of their taxonomic/phylogenetic distinctiveness, should eventually result in conservation efforts aimed at protecting them and their habitats. While the taxonomic rank of subspecies has been proposed to be of conservation utility (Phillimore and Owens 2006), it is our view that subspecies should be recognized on the basis of evidence beyond ecological/environmental singularity (e.g., cave-dwelling nature), such as discontinuities in the geographical distribution of phenotypic traits and some degree of phylogenetic distinctiveness and phylogeographic substructure. For this reason, as tempting as it is to propose subspecies names for each of the hypogean populations documented herein (with the ensuing conservation implications), we refrain from this course of action, at least until future research results in conclusive evidence in support of not only ecological but also evolutionary uniqueness. Notwithstanding this taxonomic resolve, we urge environmental policy-makers to acknowledge the ecological uniqueness of subterranean populations as diversity worth preserving.

Our discovery and documentation of hitherto taxonomically non-verified populations of hypogean *Rhamdia* highlights the continued importance of exploration in the process of documenting subterranean biodiversity, particularly in regions of the world rich with cave systems. Our findings corroborate the notion that, among Neotropical fishes, *Rhamdia* catfishes are one of the most prone and effective genera at colonizing subterranean habitats and establishing viable hypogean populations. One implication of this being that the diversity of cave-dwelling *Rhamdia* is most likely grossly underestimated, not only in Mexico but also throughout the distribution range of the genus. Consequently, much more exploration and taxonomically sound documentation work are needed to reach a more accurate picture of its hypogean diversity and ultimately about its evolutionary history.

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Biogeographical affinities of the aquatic community of Refugio Cave, a newly discovered *Astyanax* cave

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Abstract

Pachón cave in the Sierra de El Abra, in Northeast Mexico, stands out as hosting the world's most widely studied cavefish population – with over 500 scholarly articles published about the population. Refugio Cave was recently discovered in the El Abra region. This cave hosts the mysid cave shrimp *Spelaeomysis quinterensis* and the blind cave tetra fish, *Astyanax mexicanus*. This study aims to understand how the aquatic community of Refugio Cave es related to other cave populations in the area. For this purpose, the Histone *H3* gene of mysid shrimps and the *OCA2* gene that confers albinism in *Astyanax* fish was sequenced. Results support that the Refugio and Pachón aquatic communities, which are only 4.5 km away apart, are closely related. Thus, the Refugio Cave population may contribute to better understand the evolutionary history of such an important population and, perhaps, help with Pachon's cavefish conservation.

Keywords

Astyanax, Pachón Cave, Sierra de El Abra, stygobite, troglobite, troglomorphy

Introduction

The El Abra region in northeast Mexico stands out as one of the most important places for cave biology studies (Elliott 2018). The recently discovered Refugio Cave (Miranda-Gamboa et al. 2023) hosts the blind cave morph of the tetra fish, *Astyanax mexicanus*, and the mysid cave shrimp *Spelaeomysis quinterensis*. Through the sequencing of genetic markers in these two species, this study attempts to understand how the aquatic community of Refugio Cave is related to other cave populations in this area.

The blind Mexican tetra of genus *Astyanax* is ranked among the influential model systems in evolutionary developmental or EvoDevo biology (Jeffery 2001, 2012). It has also become the main contributor to understanding the genetic and developmental controls of troglomorphic features. These cavefish have a conspecific surface-dwelling morph that lives in nearby surface streams throughout most of México. Both surface and cave morphs remain inter-fertile, making the species complex and well-suited for experimental manipulations (Jeffery 2012; Elipot et al. 2014). Among multiple *Astyanax* cavefish populations, those from Pachón cave stand out as the world's most widely studied cavefish population (Keen et al. 2015). The genome from this population was the first cavefish to be sequenced (McGaugh et al. 2014; Warren et al. 2021). With fish from this population, a seminal study showed the central role of the lens in cavefish eye degeneration (Yamamoto and Jeffery 2000). The recently discovered Refugio Cave (Miranda-Gamboa et al. 2023) is only 4.5 km from Pachón cave and, thus, the closest among all caves with cavefish to this emblematic locality.

El Abra caves are also inhabited by the mysid cave shrimp *Spelaeomysis quinterensis*. Based on Histone *H3* DNA sequences of the mysids, mitochondrial DNA from *Astyanax*, and the underground hydrogeology of the region, Kopp et al. (2018) identified five biogeographic zones for the El Abra region: Sierra de Guatemala, Northern Sierra de El Abra, Central Sierra de El Abra, Southern Sierra de El Abra, and Micos (Fig. 1). The caves within these biogeographical zones share hydrologic connections (Espinasa and Espinasa 2016). It is likely that throughout their evolutionary history, cavefish populations within these zones exchanged individuals, as reflected by gene flow and population structure studies (Bradic et al. 2012). All biogeographical zones have multiple caves with *Astyanax* cavefish (Elliot 2018). The single exception is the northern Sierra de El Abra area. Pachón cave stands alone as the single described cavefish population within this biogeographic zone.

Is the "Refugio" cave within the northern Sierra de El Abra biogeographic zone, and thus, its *Astyanax* and mysid shrimp share genetic and evolutionary proximity to the Pachón community? Its geographical proximity would suggest that they could be genetically closely related. But further geographical analyses show there may be barriers for the two populations to exchange individuals. While most caves in central Sierra de El Abra are sumideros where the water goes in, and thus, the caves can share a common aquifer, on the contrary, both Pachón and Refugio Caves are independent springs with water coming out of the cave system, and do not share a common aquifer (Miranda-Gamboa et al. 2023). They would have to connect at the headwaters, and the 4.5 km distance between the two caves makes it a considerable barrier. Geology also has challenges. Between Pachón and Refugio caves, there is the remnant of a fossil canyon, which is now used by Highway 85 to Cd. Mante. This fossil canyon is almost 100 m deep (Fig. 2). Espinasa and Espinasa (2016) have argued that as tectonism elevated the El Abra region and layers above the limestone eroded away, the rivers changed their course. Around one to four mya, Río Comandante made the aforementioned canyon, and around 0.75 to three mya, as tectonism continued to elevate the area, the Río Comandante changed its route

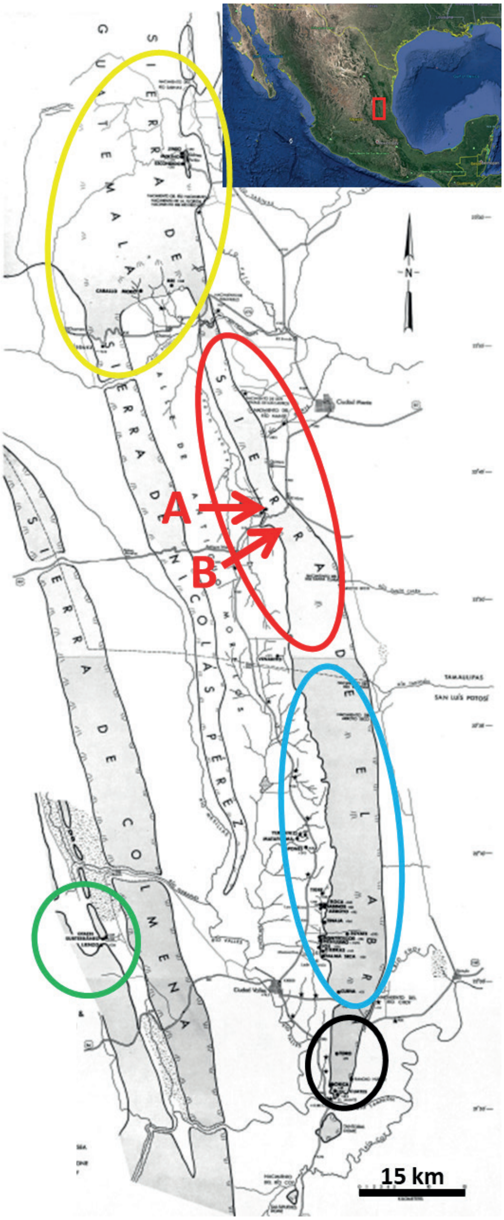

Figure 1. Map of the El Abra region, showing the five biogeographic zones proposed by Kopp et al. (2018) and supported by both *Astyanax* and mysids in this study: Sierra de Guatemala (yellow), Northern Sierra de El Abra (Red), Central Sierra de El Abra (blue), Southern Sierra de El Abra (Black), and Micos (green). **A** Pachón cave **B** Refugio Cave. Before this study, Pachón was the only known cave to harbor cavefish in the northern Sierra de El Abra. Map modified from Mitchell at al. (1977).

farther north to its current position at Servilleta canyon (Espinasa and Espinasa 2016). Several of the fossil canyons in the Sierra de El Abra have been shown to be considerable biogeographical barriers for the dispersal of aquatic organisms (Espinasa et al. 2020).

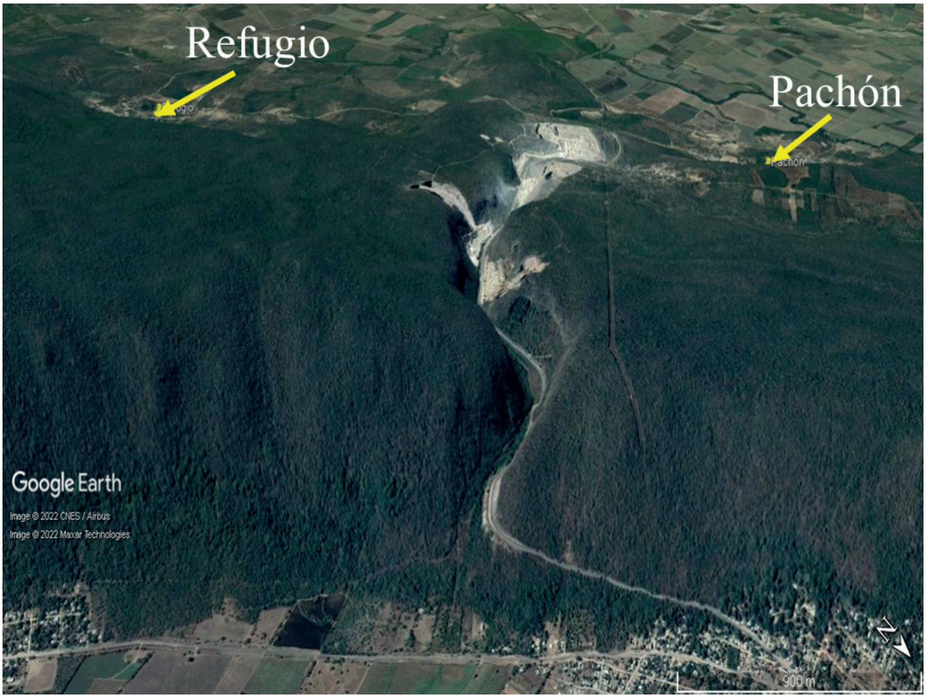

Figure 2. Between Pachón and Refugio Cave (22°34'28.50"N, 99°01'30.74"W), there is a deep fossil canyon. This canyon may be a barrier for active water connection between the two caves. Distance between Refugio and Pachón caves is 4.5 km.

While there may not be an active hydric connection between the two caves nowadays, biological communities could still be very closely related. The purpose of this study is to determine if the aquatic community inhabiting Refugio Cave is closely related to the one in Pachón. The alternate hypothesis is that the aforementioned canyon is a biogeographical barrier, and the Refugio community belongs within the Central Sierra de El Abra biogeographic zone. Two genetic markers will be used, the *Oca2* gene for *Astyanax* and the Histone *H3* for Mysid shrimps.

Most *Astyanax* cave populations are depigmented, but the Pachón population evolved albinism independently due to a unique mutation in the Ocular and Cutaneous Albinism Type II (*Oca2*) gene (Protas et al. 2006). Pachón fish have a base change (guanine instead of adenine) in exon 13 at position 1252. This unique allele can distinguish northern cavefish from the central Sierra de El Abra populations.

Mysid shrimp in central Sierra de El Abra derive from a separate lineage different from the rest of the Sierra de El Abra and Sierra de Guatemala populations (Kopp et al. 2018): When analysing the H3 marker, Pachon's mysids differ from Sierra de Guatemala populations by 7 bp (2.1%) and from central Sierra de El Abra by 31–36 bp (9.4–10.9%). The H3 sequence can be used to differentiate northern Mysids from the central Sierra de El Abra populations.

Methods

Astyanax mexicanus samples from Refugio Cave (N=11) were the same used in Miranda-Gamboa et al. (2023). Samples of *Spelaeomysis quinterensis* were collected from Refugio Cave (N=3) and Piedras Cave (N=2) in Central Sierra de El Abra. To test if the *Astyanax* Refugio population is closely related to the Pachón population, we examined if the specimens from Refugio Cave have the *Oca2* allele with the single nucleotide polymorphism (guanine instead of adenine) within exon 13 at position 1252, found in Pachón cavefish. Surface and Pachón cavefish sequences were obtained from Protas et al. (2006), Espinasa et al. (2014), and GenBank # DQ232591. To test the same hypothesis for the mysid shrimp, we used the Histone *H3* marker and compared against available sequences from Caballo Moro cave in the Sierra de Guatemala, Pachón from Northern Sierra de El Abra, Tinaja cave in Central Sierra de El Abra, and Chiquitita cave from Southern Sierra de El Abra (Kopp et al. 2018; GenBank # MH422492–MH422494). The *H3* marker has been shown to differentiate among mysid populations inhabiting different biogeographical zones of El Abra (Kopp et al. 2018). For the collection of cavefish specimens, permission was obtained from the competent Mexican authorities (SEMARNAT SGPA/DGVS/03334/22). All the fish collected were kept alive in the laboratory of Patricia Ornelas-García, IBUNAM, for breeding in captivity, which can serve as a stock for future studies.

Genomic DNA was extracted using Qiagen's DNEasy Tissue Kit by digesting a fin clip or a leg in lysis buffer. A polymerase chain reaction (PCR) amplification of a 66 bp *Oca2* gene fragment was performed using the primers 5'-GCGTATCAGGTGTCCAGAGG-3' and 5'-AGAGCATCATGGTGGTCACA-3' with an annealing temperature of 55 °C, as in Espinasa et al. (2014). For *H3*, the following primer pair was used H3aF (5' ATGGCTCGTACCAAGCAGACVGC 3') and H3aR (5' ATATCCTTRGGCATRATRGTGAC 3') with an annealing temperature of 55 °C, as in Espinasa et al. (2007). PCR amplicons were purified using the QIAquick PCR Purification Kit by QIAGEN and sent to Elim Biopharmaceuticals for sequencing. Chromatograms obtained from the automated sequencing were read, and contigs were made using the sequence editing software SequencherTM 3.0. External primers were excluded from the analyses. Sequence identity was confirmed through BLAST analyses. Sequences were aligned with ClustalW2. For mysid's *H3* sequences, the total number of bp differences among populations were counted with SequencherTM 3.0. For *Astyanax*'s *Oca2*, chromatograms were visualized at base position 1252 to determine if they were homozygous for guanine or adenine, or if they had a double peak – which is characteristic of heterozygous genotypes.

Results

The Refugio Cave entrance is at 229 masl and at the same base level as the nearby ephemeral surface stream that flows only during the rainy season. Thus, it is likely

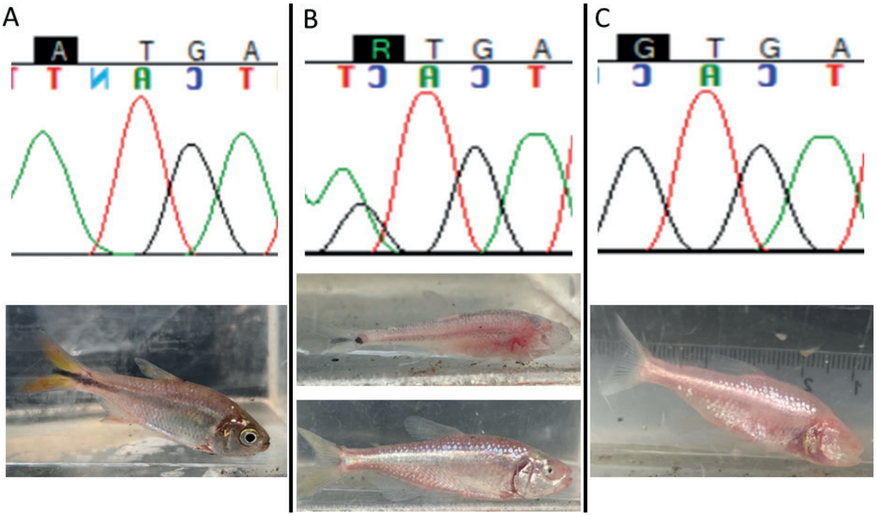

Figure 3. Chromatogram of exon 13 at position 1252 of the *Oca2* gene in Refugio Cavefish. **A** epigeomorphic fish from Refugio Cave have an adenine (green hump), just like surface fish **B** some hypothesized hybrids that show either a dark caudal spot or a dark retina, and thus are not phenotypically albino albeit being depigmented, were heterozygous, showing a distinct double green and black hump for adenine and guanine **C** the most troglomorphic fish of Refugio Cave were homozygous for guanine (Black hump), which is in the albino allele reported only for Pachón cavefish among all Sierra de El Abra populations. Fish photos modified from Miranda-Gamboa et al. (2023).

that every rainy season surface fish have direct access to El Refugio Cave (Miranda-Gamboa et al. 2023). This is unlike Pachón cave, where the entrance is at an altitude of 203 masl, perched on a steep hill with the base level of the valley at 175 masl, thus, with a strong geographic barrier preventing the influx of surface fish. As reported in (Miranda-Gamboa et al. 2023), Refugio Cave hosts an *Astyanax* mixed population of epigeomorphic fish fully pigmented, with large eyes, identical to surface fish (Fig. 3A), hybrids with either some type of eye and/or pigment (Fig. 3B), and specimens with no pigment or external remnants of eyes that look as troglomorphic as Pachón cavefish (Fig. 3C). Results also show the presence of highly depigmented individuals with eyes, and individuals with pigment but reduced eyes (Fig. 3B) as would be expected if there was introgression between the surface and cave morphs.

DNA sequences corroborated this assumption (Fig. 3). Presumptive hybrids with different degrees of eye and pigment regression were heterozygous (N=6) at base position 1252 of the surface *Oca2* allele, the most epigeomorphic fish (N=1) had the adenine found in surface fish, and the most troglomorphic fish had the guanine of the cave allele (N=4). Most relevant, these results show that some individuals of Refugio Cave have the same SNP in the *Oca2* gene as the Pachón cavefish. This SNP has not been reported in other Sierra de El Abra cavefish populations. Thus, suggesting a close relationship between Pachón and Refugio *Astyanax* populations. This information indicates that the Refugio population belongs to the Northern Sierra de El Abra biogeographical zone.

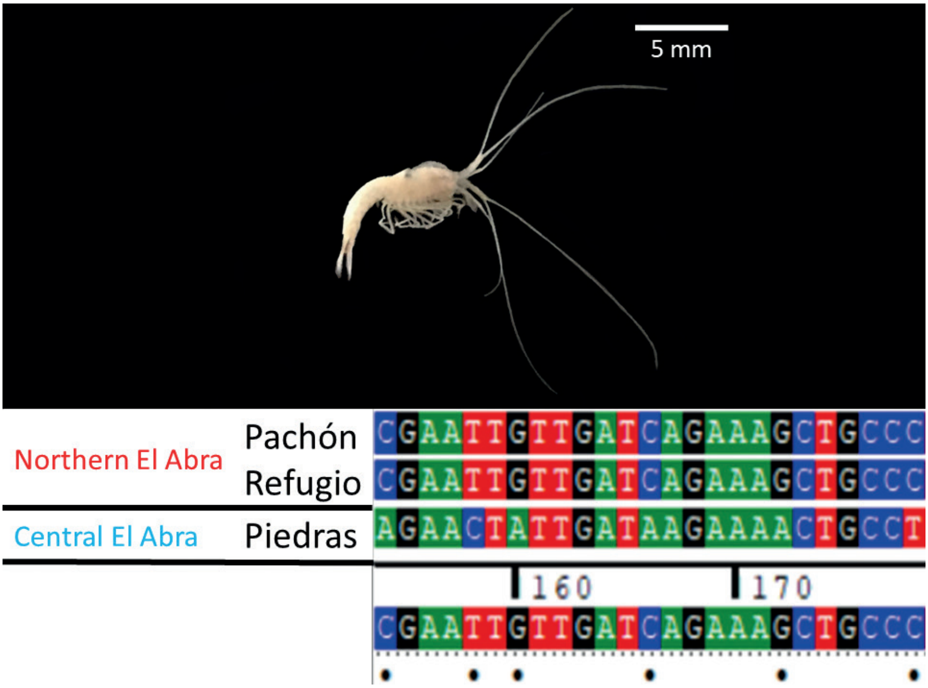

Figure 4. Mysid shrimp sequences of the Histone *H3* gene from Refugio Cave were only two bp (0.6%) different from Pachón and 34 bp (10.3%) from the Central Sierra de El Abra cave population of Piedras. Sabinos and Tinaja cave populations, also from Central El Abra, had identical sequences (not shown) to Piedras. This suggests *Spelaeomysis quinterensis* in Refugio Cave belong within the Northern Sierra de El Abra biogeographical zone and closely related to Pachón.

Mysid DNA results support that this may be true for the *Astyanax* cavefish and the entire aquatic community. The *H3* fragments were 328 bp long (GenBank # MH422492–MH422494). Mysid Refugio sequences were only two bp (0.6%) different from Pachón, 7 bp (2.1%) from Sierra de Guatemala, and 34 bp (10.3%) from the Central Sierra de El Abra cave populations of Sabinos, Tinaja, and Piedras, which had identical sequences (Fig. 4). Thus, suggesting that both aquatic species, *Astyanax* and *Spelaeomysis quinterensis* in Refugio Cave belong within the Northern Sierra de El Abra biogeographical zone (Fig. 1).

Discussion

Kopp et al. (2018) showed that the El Abra region has distinct biogeographic areas with partial barriers that affect evolutionary histories, creating evolutionary significant units for all members across distinct species of the aquatic cave community. For the Sierra de El Abra, three major biogeographic zones were delineated. A Northern, a Central, and a Southern zone. To reach this conclusion, they used hydrogeography, *H3*

DNA from the mysid Shrimp *Spelaeomysis quinterensis*, and mitochondrial DNA from *Astyanax mexicanus*. Support for such zones can also be found with nuclear markers of *Astyanax* (For ex., Bradic et al. 2012).

Our results from the mysid shrimp sequences of *H3* support that the Refugio Cave population is closely related to the Pachón population in the northern Sierra de El Abra, and very distantly related to the Central zone cave population of Sabinos, Tinaja, and Piedras (Fig. 4). Likewise, at least some Refugio *Astyanax* individuals have the same base change allele in exon 13 at position 1252 in the *Oca2* gene as in the Pachón population (Fig. 3). This allele has not been described in any other *Astyanax* populations in the Sierra de El Abra. Finally, Mitochondrial DNA sequences of Refugio *Astyanax* showed them to have the same haplotype found in the Pachón and surface populations, and not the one found in the Central Sierra de El Abra populations (Miranda-Gamboa et al. 2023). Taken together, these results suggest that the aquatic community at Refugio Cave belongs within the Northern zone (Fig. 1). It also supports that the 100 m deep remnant of a fossil canyon between the two caves (Fig. 2) – now used by Highway 85 to Cd. Mante – is not the barrier between the Northern and Southern biogeographic zones. This divide must lay further south, as proposed by Mitchell et al. (1977).

Fish from Pachón Cave stand out as the world's most widely studied cavefish population (Keen et al. 2015). Unfortunately, recent studies have suggested that its population may be declining and comprised of only a few hundred individuals (Legendre et al. 2022). With the information currently available, the Refugio Cave population appears to be the most closely related to Pachón in the entire El Abra region. Thus, the Refugio Cave population will help better understand current evolutionary forces as well as the evolutionary history of the Northern *Astyanax*. It could also serve as an alternative locality to conduct field studies, minimizing the impact on the Pachón population and helping with its conservation.

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Claudia Patricia Ornelas-García organized the expedition from which specimens from Refugio Cave were originally collected. This study was supported by the School of Sciences of Marist College. Thanks to all group members who participated in the field trip, particularly Ramses Miranda-Gamboa, Jorge Hernández-Lozano, and Jean Louis Lacaille. Proof-reading by Jordi Espinasa.

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IN MEMORIAM

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In Memoriam – Jean-Paul Mauriès

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Figure 1. Jean-Paul Mauriès in 2017: Paris, Muséum National d'Histoire Naturelle, laboratory Zoology-Arthropods. [Photo by Jean-Jacques Geoffroy.]

Jean-Paul Mauriès, a prominent myriapodologist and biospeleologist, our highly estimated colleague, and more than a good friend, passed away in Guérande (Loire-Atlantique, France) on Monday 3 October 2022 at the age of 88. He died peacefully among his close relatives after a long and unfortunately painful illness and a long time far away from his beloved specimens, collections, researches and laboratory. His funeral was held in Paris (Funerarium of the Père-Lachaise Cemetery). He is survived by his two daughters, sons-in-law and grandchildren.

Jean-Paul Mauriès-Belou was born in Albi (Tarn, France) 1st December 1934. He became a biology student at the Faculty of Sciences, University of Toulouse where he got his diploma of licence es Sciences-Naturelles (SPCN, Geology, Zoology and Botanics) in 1953–1956.

He did his military service from 1960 to 1962, partly in Algeria, and in the mean time became Assitant researcher at the CNRS from 1957 to 1966, a period during which he was more and more interested and involved in myriapod biology and systematics, notably in millipede taxonomy and distribution in the Pyrenaen area.

In 1966, he moved to Paris and became Assistant-Researcher at the Laboratory Zoology-Arthropods, Muséum national d'Histoire naturelle, directed by Prof. Max Vachon, then obtained the ranks of 'Maître-Assistant' 2nd class in 1974, 'Maître-Assistant' 1st class in 1979 and finally became 'Maître de Conférences Universitaire' in 1986 until the end of his career at the MNHN.

He payed a major role in myriapodology via his work on millipede systematics; he contributed to the original descriptions of many taxa (species, genera and families) and he rapidly became one of the main worldwide specialists of the order Chordeumatida. Beside his most exciting and passionate researches at the MNHN, he was during many years the very active curator of the collection "Myriapodes et Onychophores" of the Muséum in Paris, for which he permanently gave time for enrichment, managing and scientific updating, even after his retirement as an honorary researcher.

In 1968, he became, together with Prof. Jean-Marie Demange (Muséum, Paris) and Herr Prof.-Dr. Otto Kraus (Hamburg, Germany), a co-founder of the Centre International de Myriapodologie, the CIM, today CIM—International Society for Myriapodology. For many years, this scientific society has attracted researchers from different countries and significantly supports the study of these two groups of animals on a global scale. In collaboration with Monique Nguyen Duy – Jacquemin and Jean-Jacques Geoffroy, he contributed to the functioning of the permanent CIM—Secrétariat, Bulletin of the CIM publishing and co-organisation of international congresses of myriapodology, held every three years. Jean-Paul attended many international congresses of myriapodology (ICM), he contributed to the organization of the 1st and 9th ICM in Paris and at the end of his research career he was elected a honorary member of the CIM society.

After J.-M. Demange's retirement, he contributed also to the annual course on venomous and poisonous animals, given on millipedes and centipedes at the Muséum national d'Histoire naturelle. Jean-Paul published several papers on this topics in books dealing with the function of venom.

As a specialist of myriapod biology and systematics, his work was mainly devoted to millipede taxonomy, in France and in several European countries or regions (Switzerland, the Balkan and Iberian peninsulas, Corsica...) but also in many countries through the world: North Africa, Western Africa, Indo-Pacific, the Caribbean region, French Guiana, Brazil, Venezuela, China, South-East Asia, etc. Most of his publications are related to edaphic species in different habitats and – for many of them – to taxa strictly endemic and highly adapted to extreme environments such as high mountains but also and mostly in deep subterranean systems, MSS or caves. He contributed to subterranaen biology via the publication of review articles on troglobic myriapods in encyclopedias: "Mémoires de Biospéologie", "Encyclopaedia Biospeologica", "Encyclopaedia of Cave and Karst Science". These studies reveal the presence of species showing a great heritage and paleobiogeographic interest; some of them deserving obviously protection and conservation measures. Most of the time, the rigourous descriptions of species made by Jean-Paul were wonderfully illustrated by his own handmade drawings, witness to his undeniable scientific drawing talent.

At the very end of his life, when illness had kept him away from his researches and laboratory, the publication of very last studies in press and the expected discovery of cave-dwelling species new for France help to bring him some renewed joy and deep satisfaction for his job well done. A recent paper published by European colleagues recognizes the quality of his work by creating a new chordeumatidan genus that will honor him for ever: *Maurieseuma* Antic, Spelda, 2022. Jean-Paul Mauriès leaves us as a legacy an immense quantity of unpublished documents, such as impressive study files on diplopods of Cameroon, French Guiana and, above all, Chordeumatida.

His strong and friendly relationships with his native region of Toulouse remained intact and he published many major taxonomic papers in the journal "Bulletin de la Société d'Histoire Naturelle de Toulouse". He was an active member of several other scientific societies in which he held some various responsibilities: Société de Biogéographie, Société Zoologique de France, Société Française de Systématique, Société Française d'Ecologie, and he was during decades a member of the Société Internationale de Biospéologie – International Society for Subterranean Biology. In addition, he was an elected member of the Board of Directors of the Muséum national d'Histoire naturelle and he was for a time the temporary head of the laboratory Zoology-Arthropods.

In a completely different context, our complicit friendship was fully expressed within the CNRS choir, of which he was for years a strong pillar of the bass section. He held on the occasion of several concerts in the role of an amateur but solid soloist with a warm and deep voice. In this regard, the walls of the laboratory, which have memories, still remenber the echoes of his organ starting some traditional Occitan songs:

Quand lo boièr ven de laurar
Quand lo boièr ven de laurar
Planta son aigulhada,
A, i, ò, ú,
Planta son aigulhada, A!

We honor the memory of his wife Michèle, and give our deepest friendly regards to his two daughters Nathalie and Magali, their husbands and his grandchildren.

Today, millipedes are in mourning, they lost a good friend, as we are. This great loss will be felt very sadly by the members of the international community of myriapodologists and biospeleologists.

A selection of publications by Jean-Paul Mauriès on subterranean millipedes

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Additional paper, recently published to honor J.-P. Mauriès

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RESEARCH ARTICLE

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The first record of a stygobiotic form of *Garra rufa* (Heckel, 1843), sympatric with *Garra tashanensis* Mousavi-Sabet, Vatandoust, Fatemi & Eagderi, 2016 (Teleostei, Cyprinidae), in Iranian subterranean waters

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Abstract

We report the first finding of the stygobiotic form of the cyprinid fish *Garra rufa* (Heckel, 1843), discovered in a single locality in southwestern Iran, while the epigean form of the species is widely distributed in western Asia (Iran, Jordan, Lebanon, Turkey, and Syria). We also report a new locality for its hypogean congener, *Garra tashanensis*, about 5 km east of its type locality. The two species occur in syntopy in out-

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flows of the Tang-e-Ban, a seasonal karstic spring that only has flowing water during winter and spring, when fish individuals are washed from the cave to the surface. Identification of the investigated samples was confirmed by morphological analyses, COI distances, and a phylogenetic tree. These findings suggest the existence of a large karst aquifer in the Tashan area that harbours several cave species of fish, crustaceans, and gastropods and may have considerable conservation implications.

Keywords
Conservation, Iran, phylogeny, stygobionts, Tashan, Zagros

Introduction

Over 20 hotspots for subterranean biodiversity have been declared worldwide (Culver et al. 2021). These environments harbour a unique diversity of aquatic and terrestrial animals. Adaptations, both ecological and morphological, to the underground environment were the focus of many faunistic, evolutionary, and conservational studies (e.g., Dudich 1932; Pavan 1944; Ruffo 1957; Christiansen 1962, 2012; Sket 1985, 1999, 2004; Romero and Paulson 2001; Pipan and Culver 2012; Zagmajster et al. 2018; Borko et al. 2021) that revealed a continuum of levels (or a degree) of adaptations to underground environment.

For many species, both epigean (surface) and hypogean (subterranean) forms have been described that show variable morphological cave-related traits (Kruckenhauser et al. 2011; Pipan and Culver 2012; Kirchner et al. 2017, 2020, 2021; Bilandžija et al. 2018, 2020). These traits have been termed troglomorphisms by Christiansen (1962, 2012). Christiansen emphasised the lack of eyes and dark pigmentation but, presently, the term has been expanded to include any autapomorphy of exclusively subterranean species that may be directly related to the subterranean selective regime (Trajano and De Carvalho 2017; Culver and Pipan 2019). Troglomorphisms have been commonly separated into regressive and constructive traits (Wilkens 1988; Wilkens and Strecker 2017). Regressive traits are characterised by the loss of an organ or function, whereas constructive traits lead to an increase in the number of organs or functions, or an increase in their performance. The most prominent regressive traits in cave fishes are eye degradation and the overall reduction in pigmentation (Wilkens 1988; Jeffery 2001, 2005; Stemmer et al. 2015; Krishnan and Rohner 2016). Other morphological changes, with some related to behavioural differences, have evolved in cave fishes: for instance, the size and number of their cranial neuromasts are decreased. However, cave fishes display many other differences in morphological features (including anatomical and skeletal ones) that are not easy to interpret in terms of adaptation to subterranean habitat. In reviewing these issues, Jeffery (2001, 2005) and Yamamoto and Jeffery (2011) concluded that, in general, many regressive changes in cave fish seem to be related to loss of sight, whereas most constructive changes (although some regressive changes can be, at the same time, constructive in particular cases) seem to be related to feeding and/or swimming behaviour, and there are a few presumably neutral changes that presently defy explanation.

To date, about 300 species of cave fishes (Proudlove 2023) have been described, and of these, 64 species are from the family Cyprinidae. In particular, eight cyprinid species of the genus *Garra* Hamilton, 1822 are known troglobionts/stygobionts from South-East and South-West Asia, the Middle East, the Arabian Peninsula, and Africa. Species of *Garra* are found in fast-flowing waters such as streams and rivers, but also in lakes, springs, and caves (Krupp and Schneider 1989; Mousavi-Sabet et al. 2016; Zamani-Faradonbe and Keivany 2021). Cyprinidae with more than 63 confirmed species is the most diverse family in Iranian inland freshwater (Jouladeh-Roudbar et al. 2020). In Iran, 11 *Garra* species are known from freshwater basins (Esmaeili et al. 2016, 2017; Zamani-Faradonbe and Keivany 2021). Three of them inhabit subterranean waters in the Zagros Mountains: *Garra lorestanensis* Mousavi-Sabet & Eagderi, 2016 and *G. typhlops* (Bruun & Kaiser, 1948) occur in sympatry in Loven cave and Tuveh spring (Vatandoust et al. 2019), also together with a species from the family Nemacheilidae, *Eidinemacheilus smithi* (Greenwood, 1976) (Malek-Hosseini et al. 2022); whereas, *G. tashanensis* Mousavi-Sabet, Vatandoust, Fatemi & Eagderi, 2016 is found in Tashan cave.

Garra rufa (Heckel, 1843) is known from at least Iran, Turkey, and Syria, but so far only by its epigean (surface) form. Studies published since 2014 have provided genetic and morphological evidence for recognising some of the former subspecies and local forms of the *Garra rufa* complex as separate species, while other new species in the species complex have been described (Hamidan et al. 2014; Sayyadzadeh et al. 2015; Esmaeili et al. 2016; Mousavi-Sabet and Eagderi 2016; Mousavi-Sabet et al. 2016; Zamani-Faradonbe et al. 2020a). As a result, the range of the species is presently limited to the Tigris–Euphrates system, as well as to rivers of the Persian Gulf Basin in Iran.

The literature reports a number of morphological features that separate *Garra rufa* from its congeners in Iran and adjacent areas, such as usually 8½ branched dorsal-fin rays; the breast, belly, and predorsal mid-dorsal line fully covered by scales; eye placed in posterior half of head; the snout blunt and the head trapezoidal in dorsal view; usually 9+8 caudal-fin rays; the mental (jugular) disc fully developed; two pairs of barbels; 20–24 total gill rakers on the first branchial arch; the eyes well-developed; and a well pigmented, brown and silvery, colour pattern of the body (Ghalenoei et al. 2010; Hamidan et al. 2014; Esmaeili et al. 2016; Keivany et al. 2016; Zamani-Faradonbe et al. 2020a, b).

Here, we report the discovery of *Garra* cave fishes in Tang-e-Ban spring, five kilometres east of Tashan cave in the Zagros Mountains of southwestern Iran. Using morphology as well as COI sequence data, we show that two *Garra* species are present in sympatry in this location: one of them is the cave-restricted species *G. tashanensis* (a new record for this species), whereas the other is a novel obligate groundwater form of *Garra rufa*, a species that has so far only been recorded in surface waters.

Material and methods

Terminology

While many definitions are used in ecological and evolutionary classifications of hypogean organisms (Barr 1968; Sket 2008; Trajano and de Carvalho 2017; Culver and Pipan 2019), the objects of this study can be clearly classified, following criteria from the mentioned publications, as follows: 1. Cave fish is a generic term for fish adapted to life in caves and other underground habitats (near-synonymous terms are subterranean fish, troglomorphic fish, troglobiont, stygobiont, phreatic fish, and hypogean fish); 2. stygobiont (stygobiotic) is used for aquatic species exclusively inhabit the subterranean domain, and are unable to complete any part of their life cycle outside of subterranean habitats (obligatory cavernicole), they reproduce underground, are highly modified, and show the most profound adaptations to life in darkness.

Studied locality

Tang-e-Ban Spring is a seasonal spring (Figs 1, 2) located at 30°50'54"N, 50°13'03"E, five kilometres east of Tashan Cave (the type locality of *Garra tashanensis*), close to Ablash Village, in Tashan district, Behbahan County, Khuzestan Province. Depending on the amount of precipitation, water flows through the spring from February to May, but the spring dries out completely in summer.

Sampling

Samples were collected using a small hand net. Some specimens were photographed alive. Anaesthesia was carried out using etheric clove oil (*Eugenia caryophyllata*) diluted in water. Samples were preserved in 96% ethanol. The voucher specimens have been deposited in the Natural History Museum, Khuzestan Province (**NHMKH**), Iran and public collection of the Natural History Museum in Vienna (**NMW**), Austria. The museum numbers are given below in Examined Material.

Molecular procedures

DNA was isolated from fin clips using DNA Multisample kit (Thermo Fisher Scientific). A fragment of the mitochondrial COI gene was amplified using the primer pair FishF1 (5’-TCAACCAACCACAAAGACATTGGCAC-3’) and FishR1 (5’-TAGACTTCTGGGTGGCCAAAGAATCA-3’) (Ward et al. 2005) for some samples, and the primer pair VF2_t1 (5’-TGTAAAACGACGGCCAGTCAACCAACCACAAAGACATTGGCAC-3’) and FR1d_t1 (5’-CAGGAAACAGCTATGACACCTCAGGGTGTCCGAARAAYCARAA-3’) for other samples. PCR reactions were made in a 35 μl final reaction volume containing: 21.8 μL of H_2O, 7.1 μL of 10X DreamTaq Green Buffer, 0.5 μL of dNTP mix (10 mM each), 3.2 μL of $MgCl_2$

Figure 1. Map of Iran showing the studied localities: 1 (black): Tang-e-Ban Spring; 2 (blue): Tashan Cave; 3 (red): Sarjowshar Spring; 4 (green): Maroon River, Mooger.

(25 mM); 1 µL of each primer (20 mM), 0.2 µL of DreamTaq Green DNA Polymerase (5 U/µL) and 0.2 µL of bovine serumalbumine. PCR was performed using the following protocols: for the FishF1/FishR1 primer pair, 94 °C for 10 min; 30 cycles at 94 °C for 1 min, 58.5 °C for 1 min, 72 °C for 1 min; and a final extension for 5 min at 72 °C; for the primer VF2_t1/ FR1d_t1 primer pair: 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 52 °C for 40 s, and 72 °C for 1 min; with a final extension at 72 °C for 10 min. The PCR products were sequenced by Macrogen Europe (Amsterdam, the Netherlands). The final alignment was 649 bp in length.

Sequences (17 original ones) were assembled and checked using ChromasPro 2.1.3 (Technelysium, Tewantin, Australia). An additional 17 sequences from 15 taxa were obtained from GenBank (Suppl. material 1). Sequences were aligned using MEGA 11 (Kumar et al. 2018) (Suppl. material 2) and the same programme was used to find the best substitutional model for Bayesian and Maximum Likelihood analyses, as well as to estimate Kimura 2-parameter (K2P) pairwise distances (Kimura 1980) (Table 1). Data were curated using Mesquite version 3.7 (Maddison and Maddison 2018), then

Figure 2. Tang-e-Ban Spring in different seasons: January 2023, dry (**a**); April-May 2022 (**b–e**).

Bayesian inference of phylogenies was conducted using MrBayes v. 3.2.7a (Huelsenbeck and Ronquist 2001) using *Cyprinus carpio* Linnaeus, 1758 as an outgroup (20 million generations, four MCMC chains, sampling frequency of 1/1000). A relative burn-in was set to 25% and convergence was checked using Tracer 1.7 (Rambaut et al. 2018). Maximum likelihood phylogenetic trees with 1000 fast bootstrap replicates were obtained in IQ-TREE v2.1.3 (Minh et al. 2020). The HKY+G model of nucleotide substitution was used for both analytical methods.

Morphological analyses

Morphological analyses were based on a total of 32 absolute measurements, 45 relative measurements (ratios), 9 external body counts, and 7 axial skeleton counts (from radiographs). Measurements were made point to point using a digital caliper to the nearest 0.1 mm (only for specimens with SL>39 mm as measuring smaller fish produces significant error); counts are defined in Tables 2–5. The snout morphology and categorisation follow Nebeshwar and Vishwanath (2017). The terminology used for the external oral morphology and the gular disc (commonly referred to as mental disc or mental adhesive disc, e.g., in Hashemzadeh Segherloo et al. (2016)) followed Kottelat (2020). Measurements of the gular disc included disc length (taken as the distance between the anterior median margin of the torus and the posterior-most end of the labrum at midline); maximum mouth width (distance between lateral margins of the labelli); disc width (maximum width of the labrum); and pulvinus width (maximum width of the pulvinus).

In the description, the posterior two branched rays in the dorsal and anal fins, located on the last complex proximal pterygiophore of the fin, were symbolised as 1½ while "½" was omitted in statistical analyses. Ray counts for dorsal and anal fins were taken from radiographs. Counts and terminology of the axial skeleton, examined from radiographs, followed Naseka (1996). Total number of lateral-line scales were counted as lateral-line segments (defined by pores) as, in some phenotypes, scales are poorly developed or absent, while respective canal segments are present.

To detect separation between specimens, phenotypes, and species in the morphospace, we used both principal component analysis (PCA) and cluster analysis (CA). Statistical analyses were performed using Microsoft Excel, Statistica 12 (StatSoft), and PAST version 4.09 (Hammer et al. 2001). As measurements were only taken from specimens with SL > 39 mm (20 specimens in total), whereas counts were taken from all examined specimens (33 specimens in total), statistical analyses were performed on either morphometric and meristic characters together, or on meristic characters (i.e., counts) only.

Examined material

Identification codes used in molecular and statistical analyses are given in parentheses.

Garra rufa

Epigean *G. rufa*: NMW 100637, 9 specs (Gr1-9), SL 49.3–74.8 mm; Maroon River, Mooger 10 km to the northeast of Tang-e-Ban Spring, 20.04.2022, coll. Fatemi Y.

Hypogean phenotype of *G. rufa* (identified as such based on molecular data as shown below): 5 specs (F60-62, F64-65), SL 34.5–52.9 mm; Tang-e-Ban Spring, 20.04.2022, coll. Babolimoakher H.

Garra tashanensis, disc-bearing phenotype

Tang-e-Ban Spring (identified as *G. tashanensis* based on molecular data as shown below): NMW 100638, 1 specs (F63), 43.3 mm; 20.04.2022, coll. Babolimoakher H. (NHMKH) 12 specs (F66-75, A, B), SL 33.1–45.9 mm; SL 33.1–45.9 mm; same date and collector.

Tashan Cave: (NHMKH) 6 specs (F9, F44-46, F48, Y), SL 22.5–42.2 mm, 17.03.2018, coll. Malek-Hosseini MJ & Fatemi Y.

Comparative material: NMW 53257 (10), 53238 (4) - syntypes of *Discognathus obtusus* Heckel, 1843; NMW 53240 (8) – lectotype and paralectotypes of *Discognathus rufus* Heckel, 1843.

Results

Genetic analyses

Bayesian phylogeny placed the troglomorphic specimens from Tang-e-Ban Spring in a clade with surface *G. rufa* from a stream in Sarjowshar village about 7 km to the southwest of Tang-e-ban and with other *G. rufa* sequences from GenBank (Fig. 3). This Tang-e-Ban form, identified as *G. rufa* based on its COI sequence, is referred to as "hypogean *G. rufa*" in morphological analyses below. Specimens from another stygobiotic species, also inhabiting Tang-e-Ban Spring, were grouped with *G. tashanensis* exemplars (Fig. 3). *G. rufa* formed a well-supported clade together with *G. amirhosseini* Esmaeili, Sayyadzadeh, Coad & Eagderi, 2016, *G. elegans* (Günther, 1868), *G. mondica* Sayyadzadeh, Esmaeili & Freyhof, 2015, *G. widdowsoni* (Trewavas, 1955) and *G. persica* Berg, 1914, as well as *G. barreimiae* Fowler & Steinitz, 1956, *G. ghorensis* Krupp, 1982, *G. jordanica* Hamidan, Geiger & Freyhof, 2014, and a group of *Garra* including Lorestan cave barbs (*G. typhlops* and *G. lorestanensis*) and *G. gymnothorax* (Berg, 1949). This large clade was found to be sister to *G. tashanensis* with 0.84 posterior probability and 85% ultrafast bootstrap support.

Average estimates of genetic divergence (K2P) in the COI barcode region among the studied *Garra* samples and specimens (Table 1) revealed that hypogean *Garra rufa* of Tang-e-Ban spring shows a maximum of 1.09 % of K2P distance compared with

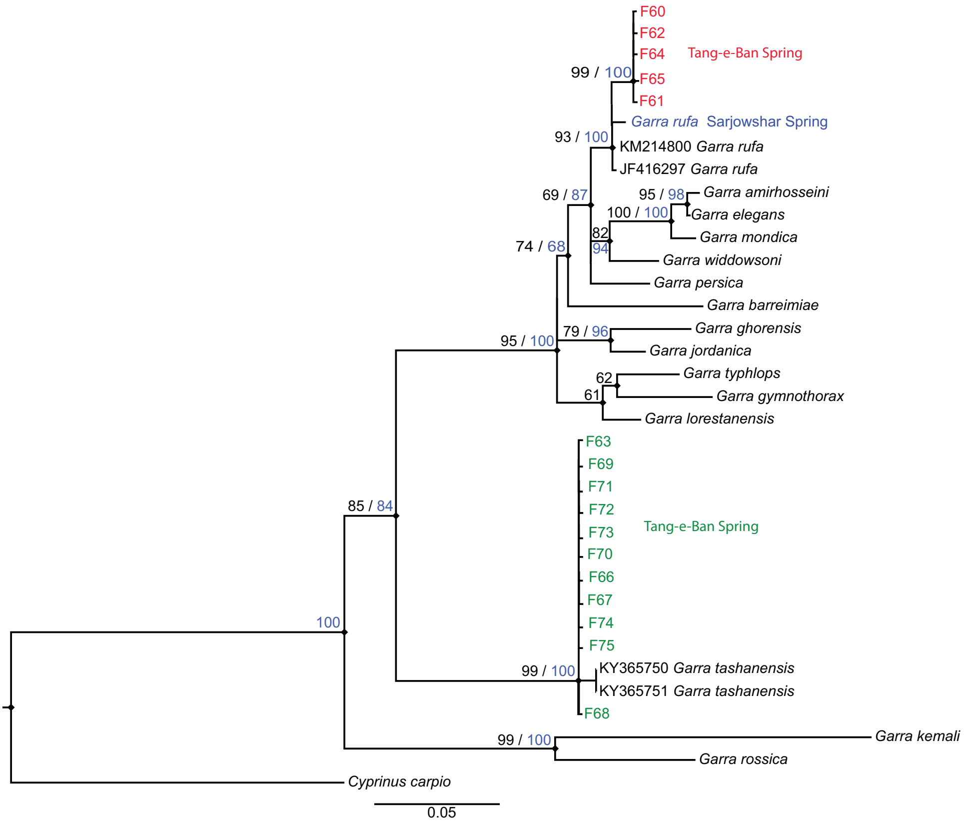

Figure 3. Phylogeny of the *Garra* lineage. The topology is from Bayesian inference analysis based on COI sequences. Blue values: posterior probabilities (Bayesian); black values: ultrafast bootstrap supports obtained by IQTREE (ML).

surface specimens from a stream in Sarjowshar village, and 0.31% with other *G. rufa* in the analyses. Analysed specimens of hypogean *G. tashanensis* from Tang-e-Ban Spring showed a maximum of 0.31% K2P distance among samples from this locality, and 0.78% K2P distance from sequences of Tashan cave individuals published by Mousavi-Sabet et al. (2016).

Morphological analyses

Garra rufa (Heckel, 1843)

Description of the cave sample (Tang-e-Ban Spring)

The general appearance of the body is shown in Figs 4, 5, morphometric data are given in Table 2 and counts are given in Table 3.

Table 1. Estimates of the average COI divergence (K2P distance) between examined *Garra* samples.* Tang-e-Ban Spring.

No.	Specimen name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
1	KM214800 Garra rufa																																	
2	JF416297 G. rufa	0.31																																
3	G. rufa Sarjowshar Spring	0.46	0.77																															
4	F60 G. rufa*	0.62	0.93	1.09																														
5	F61 G. rufa*	0.62	0.93	1.09	0.00																													
6	F62 G. rufa*	0.62	0.93	1.09	0.00	0.00																												
7	F64 G. rufa*	0.62	0.93	1.09	0.00	0.00	0.00																											
8	F65 G. rufa*	0.62	0.93	1.09	0.00	0.00	0.00	0.00																										
9	F63 G. tashanensis*	10.30	10.30	10.49	11.04	11.04	11.04	11.04	11.04																									
10	F66 G. tashanensis*	10.30	10.30	10.49	11.04	11.04	11.04	11.04	11.04	0.00																								
11	F67 G. tashanensis*	10.24	10.24	10.43	10.98	10.98	10.98	10.98	10.98	0.00	0.00																							
12	F68 G. tashanensis*	10.12	10.12	10.30	10.85	10.85	10.85	10.85	10.85	0.15	0.15	0.16																						
13	F69 G. tashanensis*	10.30	10.30	10.49	11.04	11.04	11.04	11.04	11.04	0.00	0.00	0.00	0.15																					
14	F70 G. tashanensis*	10.30	10.30	10.49	11.04	11.04	11.04	11.04	11.04	0.00	0.00	0.00	0.15	0.00																				
15	F71 G. tashanensis*	10.30	10.30	10.49	11.04	11.04	11.04	11.04	11.04	0.00	0.00	0.00	0.15	0.00	0.00																			
16	F72 G. tashanensis*	10.30	10.30	10.49	11.04	11.04	11.04	11.04	11.04	0.00	0.00	0.00	0.15	0.00	0.00	0.00																		
17	F73 G. tashanensis*	10.30	10.30	10.49	11.04	11.04	11.04	11.04	11.04	0.00	0.00	0.00	0.15	0.00	0.00	0.00	0.00																	
18	F74 G. tashanensis*	9.84	10.03	10.03	10.61	10.61	10.61	10.61	10.61	0.00	0.00	0.00	0.16	0.00	0.00	0.00	0.00	0.00																
19	F75 G. tashanensis*	10.49	10.49	10.67	11.22	11.22	11.22	11.22	11.22	0.15	0.15	0.16	0.31	0.15	0.15	0.15	0.15	0.15	0.16															
20	KY365750 G. tashanensis	10.34	10.15	10.52	11.07	11.07	11.07	11.07	11.07	0.62	0.62	0.63	0.78	0.62	0.62	0.62	0.62	0.62	0.65	0.78														
21	KY365751 G. tashanensis	10.34	10.15	10.52	11.07	11.07	11.07	11.07	11.07	0.62	0.62	0.63	0.78	0.62	0.62	0.62	0.62	0.62	0.65	0.78	0.00													
22	G. amirhosseini	4.46	4.46	4.63	5.12	5.12	5.12	5.12	5.12	10.85	10.85	10.80	10.67	10.85	10.85	10.85	10.85	10.85	10.61	11.04	10.89	10.89												
23	KM214783 G. barreimiae	6.14	6.14	5.97	6.49	6.49	6.49	6.49	6.49	12.30	12.30	12.30	12.11	12.30	12.30	12.30	12.30	12.30	12.11	12.49	12.54	12.54	7.73											
24	G. elegans	4.30	4.30	4.46	4.96	4.96	4.96	4.96	4.96	11.04	11.04	10.99	10.86	11.04	11.04	11.04	11.04	11.04	10.80	11.23	11.08	11.08	0.46	7.55										
25	G. ghorensis	5.75	5.75	6.26	6.09	6.09	6.09	6.09	6.09	9.59	9.59	9.59	9.41	9.59	9.59	9.59	9.59	9.59	9.47	9.78	10.00	10.00	7.14	8.33	6.96									
26	KX570881 G. gymnothorax	6.23	6.40	6.40	6.58	6.58	6.58	6.58	6.58	12.55	12.55	12.55	12.35	12.55	12.55	12.55	12.55	12.55	12.42	12.74	12.59	12.59	6.94	8.51	6.41	8.22								
27	G. jordanica	4.13	4.13	4.63	4.79	4.79	4.79	4.79	4.79	10.67	10.67	10.80	10.48	10.67	10.67	10.67	10.67	10.67	10.60	10.85	10.70	10.70	6.13	6.84	5.96	3.89	6.58							
28	G. kemali	15.68	15.47	15.27	15.68	15.68	15.68	15.68	15.68	14.82	14.82	15.01	14.61	14.82	14.82	14.82	14.82	14.82	15.62	15.02	14.64	14.64	16.09	17.96	16.51	17.82	16.71	17.14						
29	KM214776 G. lorestanensis	4.46	4.46	4.63	4.46	4.46	4.46	4.46	4.46	9.77	9.77	9.88	9.59	9.77	9.77	9.77	9.77	9.77	9.85	9.95	9.80	9.80	6.47	7.37	6.30	6.62	4.66	5.46	13.23					
30	G. mondica	4.64	4.64	4.83	5.41	5.41	5.41	5.41	5.41	10.84	10.84	10.84	10.63	10.84	10.84	10.84	10.84	10.84	10.84	11.06	10.86	10.86	2.00	7.52	1.45	7.00	5.62	6.19	17.11	6.59				
31	G. persica	3.01	3.01	3.49	3.66	3.66	3.66	3.66	3.66	10.54	10.54	9.89	10.35	10.54	10.54	10.54	10.54	10.54	9.47	10.72	10.57	10.57	4.65	5.97	4.81	6.27	6.58	5.98	15.76	5.31	4.83			
32	G. rossica	13.75	13.75	13.94	13.75	13.75	13.75	13.75	13.75	14.07	14.07	14.04	13.87	14.07	14.07	14.07	14.07	14.07	14.38	14.26	14.11	14.11	14.14	15.81	14.72	14.10	15.85	14.72	11.46	13.36	14.67	13.43		
33	KM214717 G. typhlops	5.29	5.29	5.13	5.29	5.29	5.29	5.29	5.29	10.84	10.84	10.97	10.66	10.84	10.84	10.84	10.84	10.84	10.79	11.03	10.88	10.88	6.65	7.56	6.82	6.28	5.01	5.63	13.25	3.64	6.99	5.82	12.20	
34	G. widdowsoni	3.32	3.32	3.80	3.64	3.64	3.64	3.64	3.64	10.85	10.85	10.80	10.67	10.85	10.85	10.85	10.85	10.85	10.80	11.04	10.89	10.89	4.30	7.01	4.13	6.96	7.11	5.62	16.30	5.29	4.07	4.48	15.31	6.47

Table 2. Morphometrics of examined *Garra rufa*.

	Hypogean sample, Tang-e-Ban			**Epigean sample, Maroon River; n=9**			
Sample label	**F60**	**F62**	**F64**	**min**	**max**	**mean**	**SD**
SL, mm	41.9	52.9	40.7	49.3	74.8	61.8	8.0
Maximum body depth (% SL)	20.1	20.3	20.2	19.8	25.5	22.5	1.7
Depth of caudal peduncle (% SL)	10.1	11.1	10.2	12.1	13.9	12.8	0.6
Depth of caudal peduncle (% length of caudal peduncle)	59.1	53.0	54.2	71.4	82.2	78.4	4.2
Body width (% SL)	15.3	15.3	15.4	14.1	17.4	15.8	1.1
Caudal-peduncle width (% SL)	8.4	8.6	8.6	7.0	9.7	8.4	0.8
Predorsal length (% SL)	51.4	51.3	51.6	45.9	50.0	48.0	1.3
Postdorsal length (% SL)	40.8	42.8	45.0	31.8	39.1	36.8	2.2
Prepelvic length (% SL)	52.9	56.2	56.6	49.8	55.2	53.4	1.8
Preanal length (% SL)	73.4	74.9	75.5	76.3	81.6	79.6	1.5
Pectoral – pelvic-fin origin length (% SL)	29.9	31.3	33.3	29.1	32.5	31.0	1.3
Pelvic – anal-fin origin length (% SL)	20.6	19.9	20.9	25.9	29.1	27.2	1.0
Caudal-peduncle length (% SL)	18.1	20.8	18.7	15.0	17.4	16.4	1.2
Dorsal-fin base length (% SL)	12.5	13.2	13.3	15.4	18.1	16.1	1.6
Dorsal-fin depth (% SL)	18.3	20.5	20.7	15.8	22.1	19.4	2.4
Anal-fin base length (% SL)	8.4	8.7	8.8	7.1	9.0	8.0	0.6
Anal-fin depth (% SL)	18.3	17.7	17.9	14.8	18.4	16.7	1.3
Pectoral-fin length (% SL)	19.7	20.9	19.1	20.8	24.3	22.9	1.3
Pelvic-fin length (% SL)	17.6	18.5	16.6	17.3	20.3	18.7	1.1
Head length (% SL)	22.9	23.5	24.0	21.9	24.1	23.3	0.8
Head length (% body depth)	113.7	116.0	118.7	92.6	121.4	104.4	9.0
Head depth at nape (% SL)	16.1	16.2	16.3	15.8	17.8	16.5	0.7
Head depth at nape (% HL)	70.2	68.9	67.9	67.1	75.9	71.0	3.1
Anus – anal-fin origin distance (% pelvic – anal-fin origin length)	32.3	28.3	30.4	24.4	31.6	27.8	2.3
Maximum head width (% SL)	17.3	17.0	17.2	16.3	18.4	17.5	0.6
Maximum head width (% HL)	75.9	72.5	71.4	68.9	78.8	75.3	3.1
Anterior barbel length (% SL)	4.3	4.2	4.8	2.8	5.4	3.8	0.8
Anterior barbel length (% HL)	52.2	40.2	51.2	30.0	42.8	35.2	4.5
Anterior barbel length (% internasal width)	82.7	66.6	66.7	38.5	68.9	50.1	9.4
Posterior barbel length (% SL)	5.5	5.2	5.2	2.1	6.3	4.2	1.2
Posterior barbel length (% HL)	19.7	22.0	21.7	9.4	27.3	17.8	5.1
Internasal width (% SL)	7.6	6.3	7.2	7.2	7.8	7.5	0.2
Internasal width (% HL)	33.2	27.0	29.8	29.9	34.9	32.1	1.6
Maximum mouth width (% HL)	42.5	40.4	39.8	40.5	48.5	45.3	3.1
Maximum mouth width (% SL)	9.7	9.5	9.6	9.0	11.5	10.6	0.9
Mouth cleft transverse length (% SL)	6.9	7.3	7.3	6.7	10.1	8.3	1.1
Mouth cleft transverse length (% HL)	30.1	30.9	30.5	30.6	42.4	35.4	4.0
Mouth cleft transverse length (% internasal width)	90.6	114.6	102.4	88.7	132.6	110.6	14.1
Disc width (% HL)	35.3	33.8	33.4	33.0	43.0	37.8	3.6
Pulvinus width (% HL)	19.7	22.0	21.7	9.4	27.3	17.8	5.1
Disk length (% disk width)	94.8	100.7	100.6	71.3	91.8	80.7	7.3
Disk length (% HL)	33.1	34.0	33.6	26.4	35.7	30.4	2.8
Width between ventral extremities of gill slits (% maximum head width)	53.9	54.1	54.0	63.1	74.8	67.6	3.8
Width between ventral extremities of gill slits (% HL)	40.9	39.2	38.6	45.3	56.9	50.9	3.5
Width between dorsal extremities of gill slits (% maximum head width)	80.4	85.9	88.3	80.8	92.2	87.7	4.4
Width between dorsal extremities of gill slits (% HL)	61.0	62.3	63.1	61.6	72.1	65.9	3.8

Longest examined specimen (F62) 52.9 mm SL (Fig. 4c). Body elongated, moderately thick, more compressed in region of caudal peduncle. Dorsal head profile rising gently, flat or slightly convex, more or less continuous with dorsal body profile to nape or about middle between nape and dorsal-fin origin. Ventral profile more or less straight to anal-fin origin. Head moderately large and markedly depressed, with slightly convex or flat interorbital distance; depth at nape considerably less than head length; width at nape exceeding head depth at nape. Snout blunt and smooth; neither

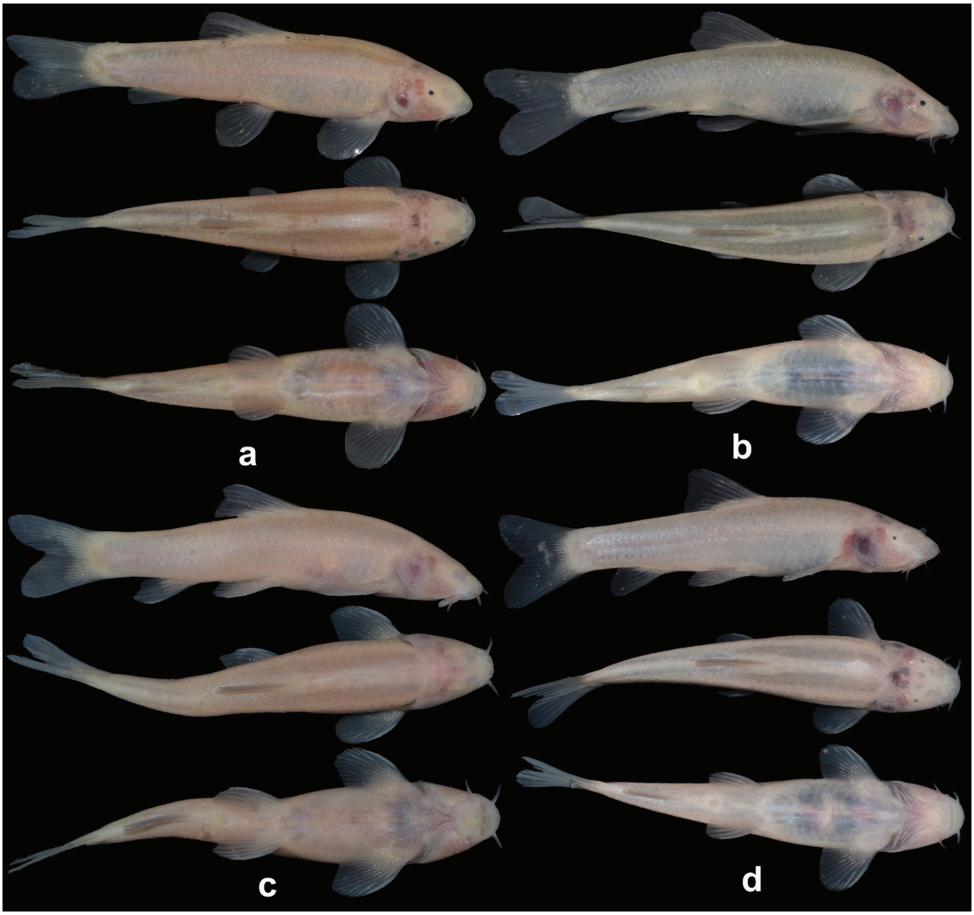

Figure 4. Hypogean *Garra rufa*, Tang-e-Ban Spring, 20.04.2022, before preservation (just anesthetized), right lateral, dorsal and ventral views: F60, SL 41.9 mm (**a**); F61, SL 35.2 mm (**b**); F62, SL 52.9 mm (**c**); and F64, SL 40.7 mm (**d**).

Table 3. Counts in examined *Garra rufa*.

	Hypogean sample, Tang-e-Ban					**Epigean sample, Maroon River; n=9**			
Sample label	**F60**	**F61**	**F62**	**F64**	**F65**	**min**	**max**	**mean**	**SD**
Number of unbranched dorsal-fin rays	3	3	3	3	4	3	4	3.8	0.4
Number of branched dorsal-fin rays (without 1/2)	7	7	7	7	7	8	8	8.0	0.0
Number of unbranched anal-fin rays	3	3	3	3	3	3	3	3.0	0.0
Number of branched anal-fin rays (without 1/2)	5	5	5	5	5	5	5	5.0	0.0
Number of simple pectoral-fin rays	1	1	1	1	1	1	1	1.0	0.0
Number of branched pectoral-fin rays	13	13	13	12	12	12	13	12.6	0.5
Number of simple pelvic-fin rays	1	1	1	1	1	1	1	1.0	0.0
Number of branched pelvic-fin rays	7	7	8	7	8	7	8	7.4	0.5
Number of predorsal vertebrae	11	11	11	11	11	10	11	10.3	0.5
Number of abdominal vertebrae	19	20	20	19	20	20	20	20.0	0.0
Number of pre-anal caudal vertebrae	2	2	2	3	2	3	5	3.8	0.7
Number of post-anal caudal vertebrae	13	14	14	13	13	11	12	11.6	0.5
Number of caudal vertebrae	15	16	16	16	15	14	16	15.3	0.7
Total vertebrae	34	36	36	35	35	34	36	35.2	0.7
Vertebrae between first pterygiophores of dorsal and anal fins	10	11	11	11	11	12	14	13.2	0.8
Number of total lateral-line scales	35	35	34	33	34	33	36	34.9	0.9

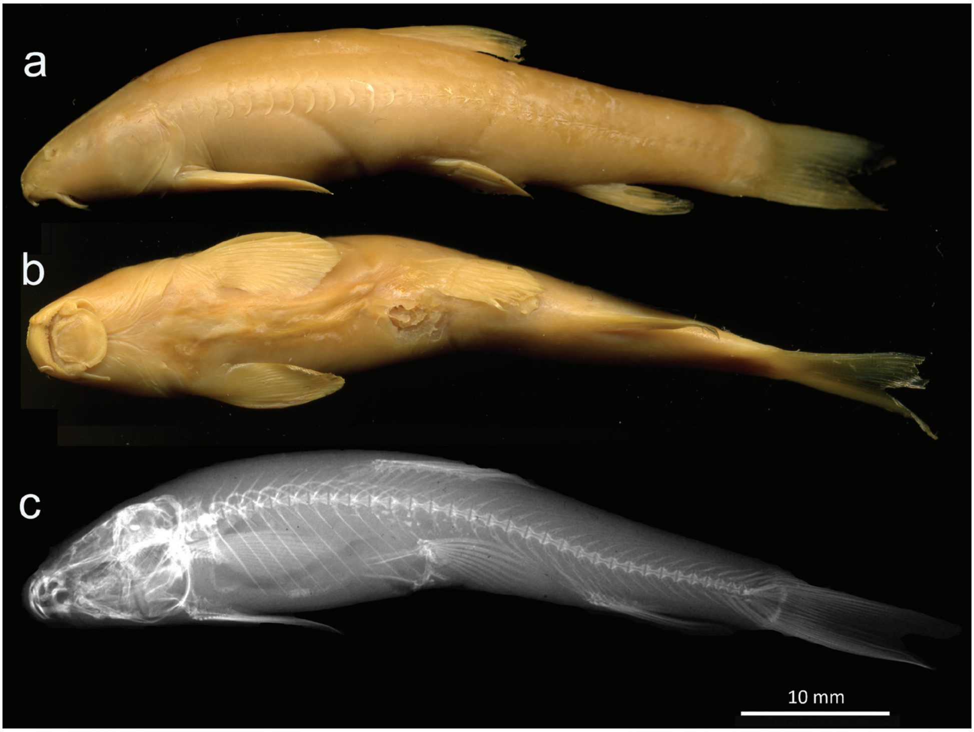

Figure 5. Hypogean *Garra rufa*, Tang-e-Ban Spring, 20.04.2022, preserved specimen (F62, SL 52.9 mm): left lateral view (**a**), ventral view (**b**) and radiograph (**c**). Radiograph showing distinguishing characters: 7½ branched dorsal-fin, 2 pre-anal caudal vertebrae, 14 post-anal caudal vertebrae, and 11 vertebrae between first pterygiophores of dorsal and anal fins.

transverse groove nor transverse lobe developed. Anterior extremity of ethmoid field (proboscis) not elevated from depressed rostral surface. Tubercles absent.

Eye variably reduced from almost "normal" eye to complete lack of externally visible structures; reduction asymmetrical (in 4 specimens), as follows, by specimens (left / right side of head). F60: eye pigmented; fossa small / eye pigmented; fossa small; F61 (Fig. 4b): eye slightly pigmented; fossa very small / eye pigmented; fossa comparatively large; F62 (Fig. 4c): no pigmented eye; fossa very small / no pigmented eye; no fossa; F 64 (Fig. 4d): eye pigmented; fossa very small / eye pigmented; fossa very small; F 65: eye pigmented; no fossa / eye pigmented; fossa small.

Gular disc well-developed, with free lateral and posterior margins, roundish, its width about equal to length; no considerable variability of its size and shape found in examined specimens. Mouth inferior, mouth cleft clearly straight. Papillae on torus, labellum and labrum. Rostral cap well-developed, fimbriate, papillate on ventral surface. Upper jaw almost or completely covered by rostral cap. Barbels in two pairs; anterior barbel well-developed, long; posterior barbel at corner of mouth, variably longer than rostral barbel.

Dorsal fin with 3 in 4 specimens and 4 in one specimen simple and 7½ branched rays; outer dorsal-fin margin about straight or slightly concave; origin at about middle of body, inserted anterior to vertical from pelvic-fin origin; first branched ray longest. Pectoral fin with 1 simple and 12–13 branched rays, depth less than head length. Pelvic fin with 1 simple and 7–8 branched rays, origin closer to anal-fin origin than to pectoral-fin origin, inserted below second or third branched dorsal-fin ray. Anal fin with 3 simple and 5½ branched rays; first branched ray longest; distal margin slightly to markedly concave; origin closer to caudal-fin base than to pelvic-fin origin. Distance between anus and anal-fin origin about equal to one third of pectoral – pelvic-fin origin length. Caudal fin forked with Caudal fin forked with 2+17(9+8) principal rays.

Body variably naked. Most scales lacking except for complete or almost complete lateral line with 33–35 total scales (Fig. 5). Besides lateral-line scales, few scattered or more numerous overlapping scales present on sides of trunk and caudal peduncle in 4 specimens out of 5 examined: 9–19 above lateral line and 2–12 below lateral line. Lateral-line scales comparatively well ossified, visible without staining with Alizarin Red S along most lateral line except for terminal section where lateral canal still well seen by normally developed sensory pores. Cephalic sensory canals complete, fully developed, non-interrupted.

Total vertebrae 34(1), 35(2) or 36(2); abdominal vertebrae 19(2) or 20(3); predorsal abdominal vertebrae 11; caudal vertebrae 15 (including 2 pre-anal and 13 post-anal caudal vertebrae) or 16 (2+14 in 2 specimens and 3+13 in one); and 10(1) or 11(4) vertebrae between first pterygiophores of dorsal and anal fins.

Body and fins unpigmented; body of live specimens (Fig. 4) pinkish because of blood vessels seen through the semi-transparent skin. In preserved specimens, body turns yellowish-white.

Comparison with epigean sample of *Garra rufa*

The sample examined from Maroon River did not depart from "typical" *G. rufa* morphology. We did not specifically analyse morphometric differences of the Maroon sample from *G. rufa* in other Iranian localities. The morphometric parameters of this species are age-and-size dependent and may be also influenced by habitat parameters (see, e.g., Zamani-Faradonbe et al. 2020a, b, Zamani-Faradonbe and Keyvani 2021). However, the diagnostic features of the species (such as most count characters and the gular disc shape) were shared by the Maroon sample and the examined type material of *G. rufa* and *G. obtusa* (a synonym of *G. rufa*). They are as follows: breast, belly, predorsal and mid-dorsal line fully covered by scales; lateral line complete, with 33–36 total lateral-line scales; snout blunt and head trapezoidal in dorsal view; jugular disc fully developed, wider than long; two pairs of barbels; eyes well-developed; brown and silvery colour pattern; 9+8 caudal-fin rays; commonly 4 unbranched dorsal-fin rays; 8½ branched dorsal-fin rays; 34–36 total vertebrae; 20 abdominal vertebrae; 10–11 predorsal abdominal vertebrae; 14–16 caudal vertebrae (including 3–5 pre-anal and 11–12 post-anal caudal vertebrae); and 12–14 vertebrae between first pterygiophores of dorsal and anal fins (Tables 2, 3).

A comparison of the examined epigean and hypogean samples revealed some clear differences between them. Although some morphometric differences may be due to the fact that the hypogean specimens (SL of morphometrically examined specimens was 40.7–52.9 mm) were smaller than the epigean ones (SL 49.3–74.8 mm), the differences still deserve attention as they corroborate differences in some meristic characters. Among the morphometric parameters, the most statistically significant differences (the results of the statistical analyses are presented below) were found in the following relative measurements (Table 2): depth of caudal peduncle (10–11% SL and 53–59% of caudal peduncle length for hypogean specimens vs. 12–14 and 71–82 for epigean ones); caudal peduncle length (18–21% SL vs. 15–17); predorsal length (51–52% SL vs. 46–50); postdorsal length (41–45% SL vs. 32–39); pre-anal length (73–75% SL vs. 76–82); pelvic to anal-fin origin length (20–21% SL vs. 26–29); dorsal-fin base length (13% SL vs. 15–18); distance between ventral extremities of gill slips (39–41% HL vs. 45–57); gular disc length (95–101% of disc width vs. 71–91), meaning gular disc about as long as wide in hypogean sample (Fig. 5) in contrast to markedly wider than long in the epigean form (Fig. 6). Relative head length and most relative measurements on the head (Table 2) were similar in the two samples except for the mentioned gular disc parameters and the distance between the ventral extremities of the gill slips.

Among the examined morphometric characters, the most prominent differences were 7½ branched dorsal-fin rays in the hypogean sample (vs. 8½ in the epigean fish); 2 or 3 (in one specimen only) pre-anal caudal vertebrae (vs. 3–5, commonly 4); 13–14 post-anal caudal vertebrae (vs. 11–12); and 10–11 vertebrae between first pterygiophores of dorsal and anal fins (vs. 12–14) (Tables 2, 3). These characters entail some morphometric difference (difference in external characters) described above, namely, a shorter base of the dorsal fin when compared to the epigean specimens with 8½ branched dorsal-fin rays, and, as a result, different predorsal and postdorsal distances. The difference in the structure of the caudal vertebral region (2+[13–14] vs. 4+[11-12]) determines not only the difference in the number of vertebrae between first pterygiophores of dorsal and anal fins (pre-anal caudal subregion is shorter and the post-anal subregion is longer in the hypogean fish) but also the position of the anal fish externally expressed through, e.g., pre-anal distance and length of caudal peduncle.

The hypogean and epigean samples were clustered in distinct groups in the CA (Fig. 7) and formed well-separated groups in the morphospace using PCA using the combination of morphometric and meristic characters, as well as when using only meristic characters (Figs 7a, 8a). Interestingly, that in the CA, the hypogean *G. rufa* sample

Figure 6. Epigean (surface) *Garra rufa*, Maroon River, Mooger, 10 km to the northeast of Tang-e-Ban Spring, 20.04.2022.

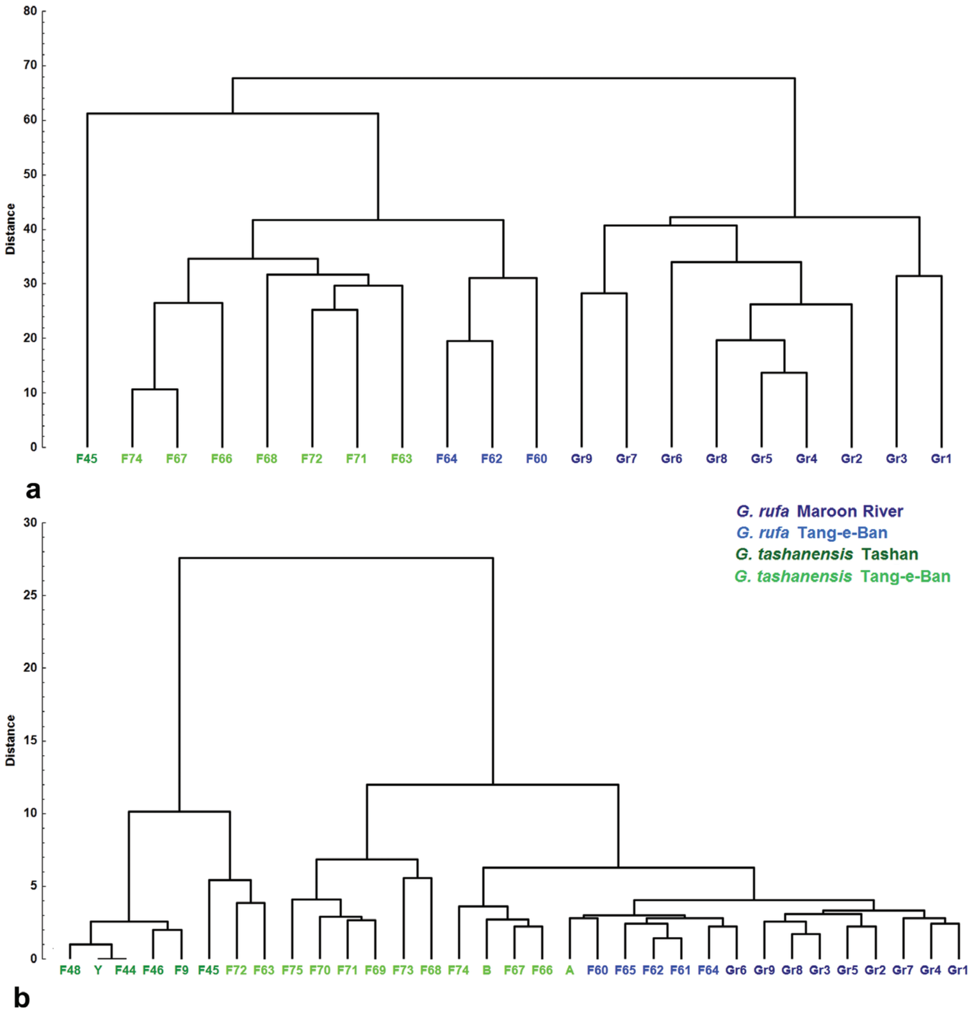

Figure 7. Results of CA: using morphometric and meristic characters (total of 20 specimens) (**a**); using only meristic characters (total of 33 specimens) (**b**).

clustered together with the syntopic *G. tashanensis*, not with epigean *G. rufa*, occurring nearby. When only meristic characters were used, the two groups were much closer in PCA (Fig. 7b) and, in CA (Fig. 8b), and even clustered together with one specimen of epigean *G. rufa* and one specimen of Tang-e-Ban *G. tashanensis.*

Garra tashanensis Mousavi-Sabet, Vatandoust, Fatemi & Eagderi, 2016

Description of the Tang-e-Ban Spring disc-bearing form

The general appearance of the body is shown in Figs 9–11, morphometric data are given in Tables 4, 5.

Table 4. Morphometrics of examined *Garra tashanensis* (SL>39.0 mm).

	Disc-bearing phenotype, Tang-e-Ban, 7 specs.									Disc-bearing phenotype, Tashan Cave (type-locality)
	F63	**F66**	**F67**	**F68**	**F71**	**F72**	**F74**	**mean**	**SD**	**F45**
SL, mm	43.3	45.9	41.4	39.5	41.5	41.4	40.1	41.9	2.1	42.2
Maximum body depth (% SL)	18.2	22.0	18.7	21.6	18.4	19.9	19.8	19.8	1.5	24.3
Depth of caudal peduncle (% SL)	11.2	13.3	11.9	12.1	11.3	12.5	11.8	12.0	0.7	12.0
Depth of caudal peduncle (% length of caudal peduncle)	55.0	64.2	61.0	69.2	55.1	66.6	59.5	61.5	5.5	79.7
Body width (% SL)	16.0	16.5	15.2	16.4	14.3	16.1	15.7	15.8	0.8	20.1
Caudal-peduncle width (% SL)	8.1	8.3	7.7	7.3	7.7	7.5	8.0	7.8	0.4	8.6
Predorsal length (% SL)	56.7	55.6	53.2	56.2	54.9	53.4	53.1	54.7	1.5	51.5
Postdorsal length (% SL)	37.6	40.3	36.9	37.7	35.4	37.9	38.5	37.8	1.5	36.7
Prepelvic length (% SL)	58.2	59.2	56.2	56.9	56.3	57.4	56.5	57.2	1.1	56.0
Preanal length (% SL)	80.4	82.0	77.4	78.0	77.6	78.6	78.2	78.9	1.7	76.6
Pectoral – pelvic-fin origin length (% SL)	32.6	36.8	34.3	33.5	33.1	35.1	33.8	34.2	1.4	31.6
Pelvic – anal-fin origin length (% SL)	21.8	21.9	19.2	20.9	19.3	17.7	19.6	20.1	1.5	20.5
Caudal-peduncle length (% SL)	20.3	20.8	19.5	17.5	20.4	18.8	19.8	19.6	1.1	15.0
Dorsal-fin base length (% SL)	10.5	10.3	10.6	10.7	12.2	9.9	9.8	10.6	0.8	12.3
Dorsal-fin depth (% SL)	18.5	19.3	17.3	18.7	16.8	17.4	18.4	18.1	0.9	20.9
Anal-fin base length (% SL)	7.9	8.1	7.7	7.5	7.4	8.1	7.7	7.8	0.3	6.8
Anal-fin depth (% SL)	16.6	17.0	15.8	15.2	14.9	17.0	16.2	16.1	0.8	14.8
Pectoral-fin length (% SL)	16.8	18.2	18.9	16.7	16.8	17.6	18.6	17.7	0.9	20.9
Pelvic-fin length (% SL)	14.8	14.6	14.9	15.3	14.3	14.4	16.1	14.9	0.6	16.7
Head length (% SL)	25.3	24.7	24.9	25.3	24.5	24.7	25.5	25.0	0.4	28.0
Head length (% body depth)	138.6	112.2	133.0	116.9	133.3	124.2	129.0	126.7	9.5	115.3
Head depth at nape (% SL)	13.6	14.8	13.9	15.1	14.2	14.6	14.1	14.3	0.5	18.2
Head depth at nape (% HL)	53.6	59.9	55.7	59.9	57.9	59.0	55.3	57.3	2.5	65.1
Anus – anal-fin origin distance (% pelvic – anal-fin origin length)	30.1	32.8	27.9	23.3	32.8	30.2	30.0	29.6	3.3	18.9
Maximum head width (% SL)	18.4	19.0	19.3	20.0	19.4	18.9	19.4	19.2	0.5	21.7
Maximum head width (% HL)	72.8	76.9	77.3	79.0	79.3	76.5	76.1	76.8	2.2	77.6
Anterior barbel length (% SL)	5.6	5.6	5.5	6.2	5.5	5.7	5.3	5.6	0.3	7.2
Anterior barbel length (% HL)	45.7	44.1	48.4	50.2	49.2	48.9	48.9	47.9	2.2	42.3
Anterior barbel length (% internasal width)	72.7	83.2	83.1	74.9	18.4	19.9	79.6	77.1	4.8	98.7
Posterior barbel length (% SL)	8.2	6.7	6.6	7.3	6.8	6.7	6.4	6.9	0.6	4.0
Posterior barbel length (% HL)	32.3	27.2	26.4	28.9	27.7	27.0	25.1	27.8	2.3	14.1
Internasal width (% SL)	7.7	6.7	6.6	8.3	7.6	7.7	6.7	7.3	0.7	7.3
Internasal width (% HL)	30.4	27.3	26.4	32.8	31.1	31.3	26.3	29.4	2.7	26.0
Maximum mouth width (% HL)	41.8	45.1	39.2	40.0	37.4	40.0	41.6	40.7	2.4	50.8
Maximum mouth width (% SL)	10.6	11.1	9.8	10.1	9.2	9.9	10.6	10.2	0.7	14.2
Mouth cleft transverse length (% SL)	7.6	7.9	7.1	7.4	6.9	7.2	7.5	7.4	0.3	8.4
Mouth cleft transverse length (% HL)	30.0	31.9	28.6	29.4	28.1	29.1	29.4	29.5	1.2	30.1
Mouth cleft transverse length (% internasal width)	98.5	116.8	108.5	89.6	90.5	92.8	111.9	101.2	11.1	116.0
Disc width (% HL)	32.2	34.6	30.4	30.4	29.4	30.9	32.0	31.4	1.7	43.7
Pulvinus width (% HL)	32.3	27.2	26.4	28.9	27.7	27.0	25.1	27.8	2.3	14.1
Disk length (% disk width)	92.6	91.6	93.9	90.8	91.0	99.1	91.4	92.9	2.9	70.8
Disk length (% HL)	29.9	31.7	28.6	27.6	26.7	30.6	29.2	29.2	1.7	31.0
Width between ventral extremities of gill slits (% maximum head width)	43.4	49.4	43.5	34.4	45.7	52.6	46.0	45.0	5.7	36.1
Width between ventral extremities of gill slits (% HL)	31.6	38.0	33.6	27.2	36.2	40.2	35.0	34.5	4.3	28.0
Width between dorsal extremities of gill slits (% maximum head width)	68.9	75.7	71.4	66.4	72.6	73.4	70.6	71.3	3.0	70.8
Width between dorsal extremities of gill slits (% HL)	50.1	58.2	55.2	52.5	57.5	56.2	53.7	54.8	2.9	54.9

Longest examined specimen 45.9 mm SL (F66, Fig. 9b). Body shape considerably variable (Figs 9, 10a–c). Head slightly to markedly (Figs 9a, 10c) depressed, its transition to back with nuchal hump especially prominent in specimens with depressed head.

Table 5. Counts in examined *Garra tashanensis*.

	Disc-bearing phenotype, Tang-e-Ban Spring, 13 specs (SL 33.1-45.9 mm)				**Disc-bearing phenotype, Tashan Cave (type-locality)**					
	min	**max**	**mean**	**SD**	**F9**	**F45**	**F44**	**F46**	**F48**	**Y**
SL, mm	33.1	45.9	38.8	4.0	34.8	42.2	26	25.1	24.5	22.5
Number of unbranched dorsal-fin rays	2.0	3.0	2.2	0.4	3	3	3	3	3	3
Number of branched dorsal-fin rays (without 1/2)	7.0	7.0	7.0	0.0	7	7	7	7	7	7
Number of unbranched anal-fin rays	2.0	2.0	2.0	0.0	2	2	2	2	2	2
Number of branched anal-fin rays (without 1/2)	5.0	5.0	5.0	0.0	5	5	5	5	5	5
Number of simple pectoral-fin rays	1.0	1.0	1.0	0.0	1	1	1	1	1	1
Number of branched pectoral-fin rays	11.0	14.0	12.1	0.9	14	13	13	14	14	13
Number of simple pelvic-fin rays	1.0	1.0	1.0	0.0	1	1	1	1	1	1
Number of branched pelvic-fin rays	6.0	8.0	7.1	0.7	7	7	7	7	7	7
Number of predorsal vertebrae	11.0	13.0	11.7	0.6	10	10	10	10	10	10
Number of abdominal vertebrae	18.0	19.0	18.5	0.5	17	18	18	18	18	18
Number of pre-anal caudal vertebrae	3.0	4.0	3.1	0.3	3	2	3	2	3	3
Number of post-anal caudal vertebrae	12.0	14.0	12.8	0.7	12	13	13	13	13	13
Number of caudal vertebrae	15.0	17.0	15.9	0.6	15	15	16	15	16	16
Total vertebrae	33.0	36.0	34.5	0.8	32	33	34	33	34	34
Vertebrae between first pterygiophores of dorsal and anal fins	9.0	11.0	9.8	0.7	10	10	11	10	11	11
Number of total lateral-line scales	10.0	34.0	23.9	7.5	0	7	0	0	0	0

Predorsal back outline markedly rising and going parallel to ventral profile or sloping to dorsal-fin origin. Thus, body deepest in front of dorsal-fin origin. Pelvic fin origin below middle of dorsal-fin base. Caudal peduncle moderately deep (11–13% SL) and its depth varying within wide range of 55–69% of caudal-peduncle length. Head not deep (head depth at nape 53–60% HL), its length (24–26% SL) considerably exceeding maximum body depth (18–22% SL). Snout variably elongated, markedly arched in dorsal or ventral (Fig. 11a–e) view; neither transverse groove nor transverse lobe developed. Anterior extremity of ethmoid field (proboscis) slightly to markedly (Fig. 10c) elevated from rostral.

Eye absent; no eye fossa in examined specimens. Gular disc (Fig. 11a–e) well-developed in all specimens examined, slightly wider than long or disc length almost equal to disc width, with posterior margin considerably variable, truncate (Fig. 11e), roundish (Fig. 11a, c) or attenuated (Fig. 11b, d). Width of pulvinus about equal to disc length. Maximum width of mouth considerably exceeding disc length. Mouth inferior, mouth cleft clearly arched. Small papillae on torus, labellum and labrum. Rostral cap moderately wide, not completely covering upper lip and upper jaw (Fig. 11a–e), with markedly arched (in ventral aspect) slightly fimbriate distal margin. Anterior barbel shorter than posterior barbel.

Dorsal fin with 2 or 3 (found in 2 specimens only) unbranched and 7½ branched rays, anal fin with 2 unbranched and 5½ branched rays. Pectoral fin with 1 unbranched ray and 11 (3), 12 (7), 13 (3), or 14 (1) branched rays. Pelvic fin with single unbranched ray and 6–8 branched rays.

Body naked except for lateral line and (in three specimens) few (1–6) scattered scales behind opercule or further caudad right above or below lateral line. Lateral line

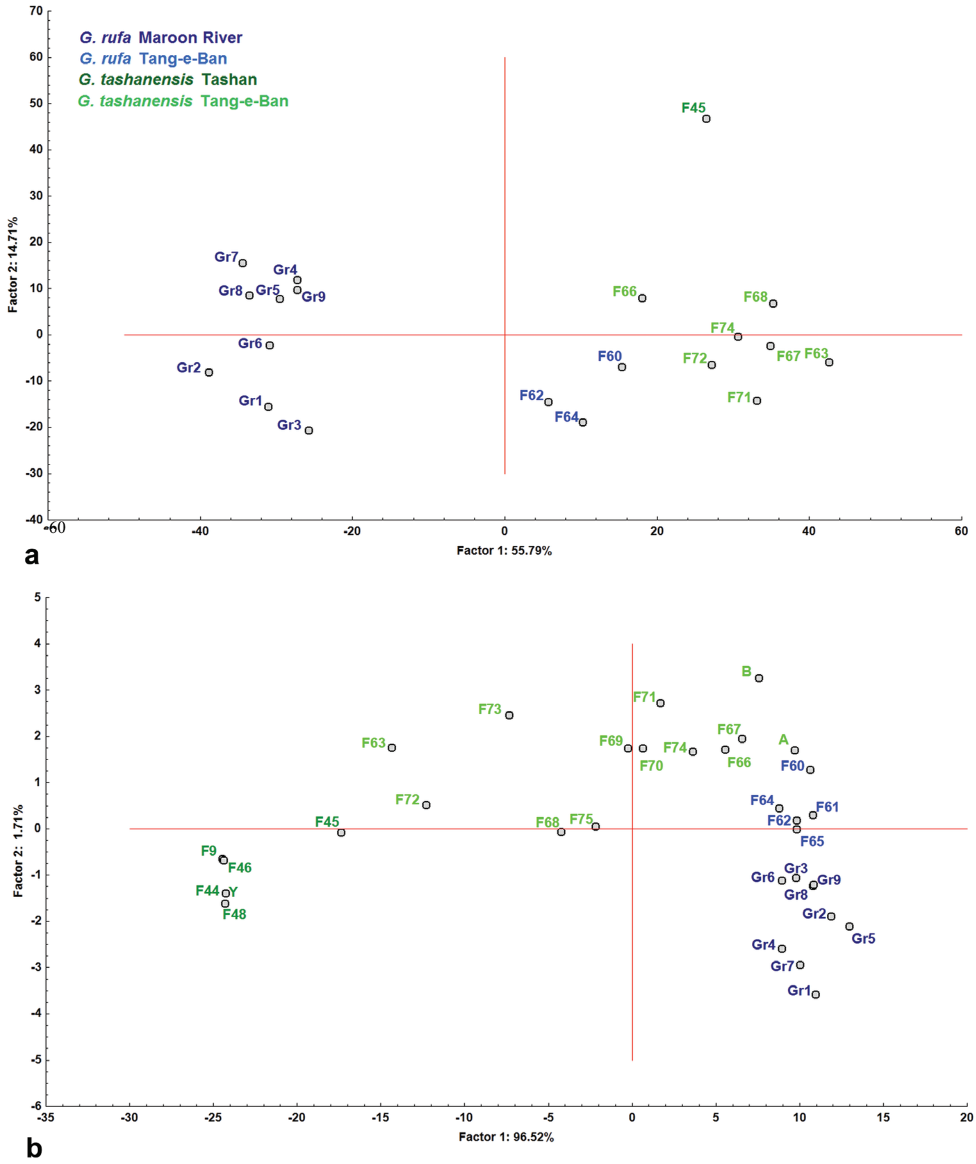

Figure 8. Results of PCA: using morphometric and meristic characters (total of 20 specimens) (**a**); using only meristic characters (total of 33 specimens) (**b**).

present in all examined specimens, variably shortened and interrupted, but commonly long or almost complete (in one specimen), with 10–34 (averaging 24) scales. Cephalic sensory canals complete, fully developed, non-interrupted.

Total vertebrae 33 (1), 34 (6), 35(5) or 36 (1); abdominal vertebrae 18(6) or 19 (7); predorsal abdominal vertebrae 11 (4), 12 (7) or 13 (1); caudal vertebrae 15(3), 16(8) or 17 (2); pre-anal caudal vertebrae 3 (4 in single specimen), post-anal caudal

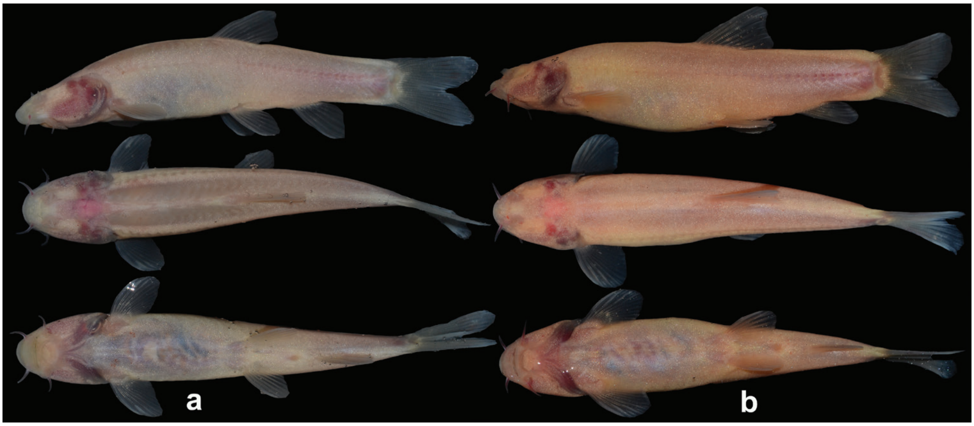

Figure 9. *Garra tashanensis*, Tang-e-Ban Spring, 20.04.2022, before preservation (just anesthetized), left lateral, dorsal and ventral views: F63, SL 43.3 mm (**a**); F66, SL 45.9 mm (**b**).

vertebrae 12 (4), 13 (7) or 14 (2); and 9(5), 10(6) or 11(2) vertebrae between first pterygiophores of dorsal and anal fins.

Body and fins unpigmented; in live specimens (Fig. 9) body is pinkish because of blood vessels seen through the semi-transparent skin. In preserved specimens, body is yellowish-white.

Description of the Tashan (type locality) disc-bearing phenotype

Measurements of one larger specimen and counts for all examined specimens are given in Tables 4, 5.

Longest examined specimen 42.2 mm SL (Fig. 10d). Body deep, thick; dorsal head profile slightly convex, its transition to back smooth, slight nuchal hump only in longest specimen. Predorsal back outline rising gently, slightly convex, to dorsal-fin origin. Pelvic-fin origin below middle of dorsal-fin base or slightly behind. Caudal peduncle deep and short, its depth varying within wide range of 80% of caudal-peduncle length. Head large (head length 28% SL) slightly exceeding maximum body depth (24% SL). Head wide and relatively deep; (head depth at nape 65% HL). Snout blunt and smooth; neither transverse groove nor transverse lobe developed. Anterior extremity of ethmoid field (proboscis) slightly elevated from rostral surface only in the longest specimen.

Eye absent; no eye fossae in examined specimens. Gular disc well-developed in all specimens examined (including smallest ones, SL 22.5–26 mm), wider than long (Fig. 11f), with roundish posterior margin. Width of pulvinus less than disc length. Maximum width of mouth considerably exceeding disc length. Mouth inferior, mouth cleft clearly straight. Small papillae on torus, labellum, and labrum. Rostral cap wide, completely covering upper lip and upper jaw (Fig. 11f), with almost straight (in ventral aspect) considerably fimbriate distal margin. Anterior barbel longer than posterior barbel. Dorsal fin with 3 unbranched and 7½ branched rays, anal fin with 2

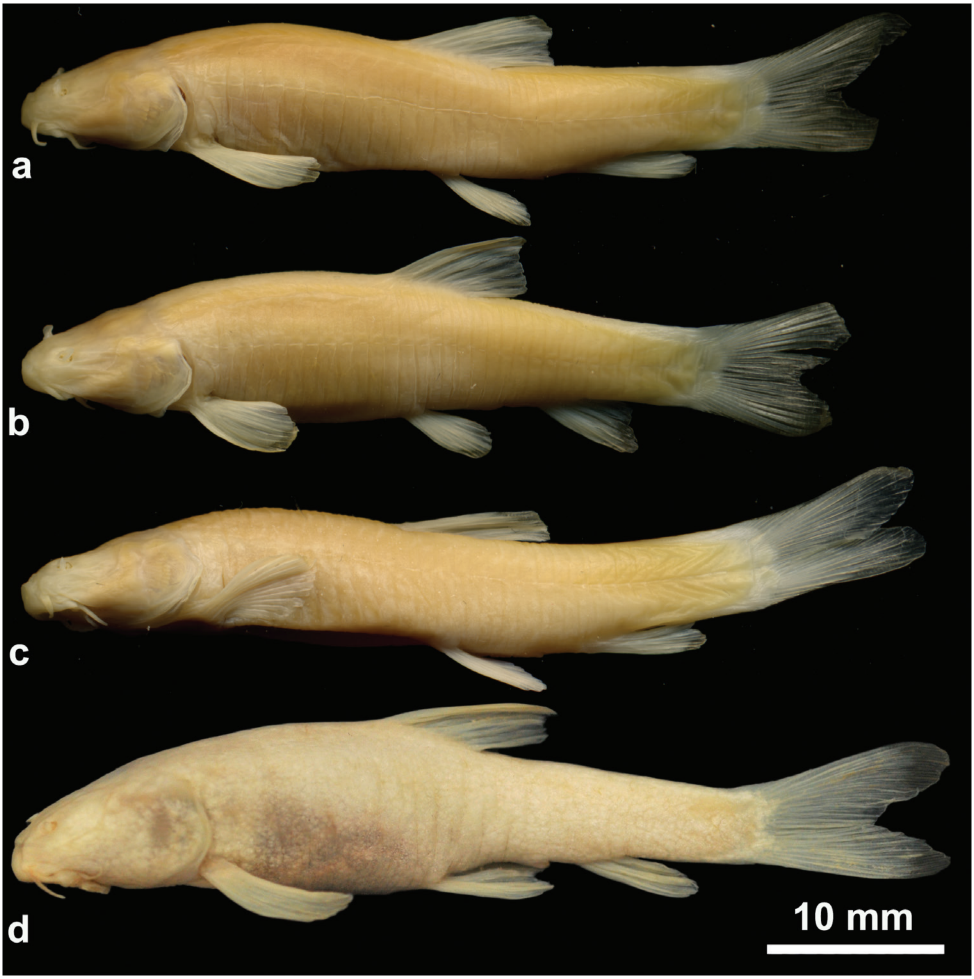

Figure 10. *Garra tashanensis*, left lateral view: F67, SL 41.4 mm (**a**); F68, SL 39.5 mm (**b**); F72, SL 41.6 mm (**c**); and F45, SL 42.2 mm (**d**). Tang-e-Ban Spring, 20.04.2022 (**a–c**) and Tashan Cave (type-locality), 17.03.2018 (**d**).

unbranched and 5½ branched rays. Pectoral fin with 1 unbranched ray and 13(3) or 14(3) branched rays. Pelvic fin with single unbranched ray and 7 branched rays.

Body naked. Lateral line absent (in specimen up to SL 34.8 mm) or present by 7 segments behind head (in longest examined specimen of SL 42.2. mm). Cephalic sensory canals complete, fully developed, non-interrupted. Total vertebrae 32 (1), 33(2), or 34 (3); abdominal vertebrae 17(1) or 18 (5); predorsal abdominal vertebrae 10; caudal vertebrae 15(3) or 16(3); pre-anal caudal vertebrae 2(2) or 3 (4), post-anal caudal vertebrae 12 (1) or 13 (5); and 10(3) or 11 (3) vertebrae between first pterygiophores of dorsal and anal fins.

Body and fins unpigmented; in live specimens, body is pinkish because of blood vessels seen through the semi-transparent skin. In preserved specimens, body is yellowish-white.

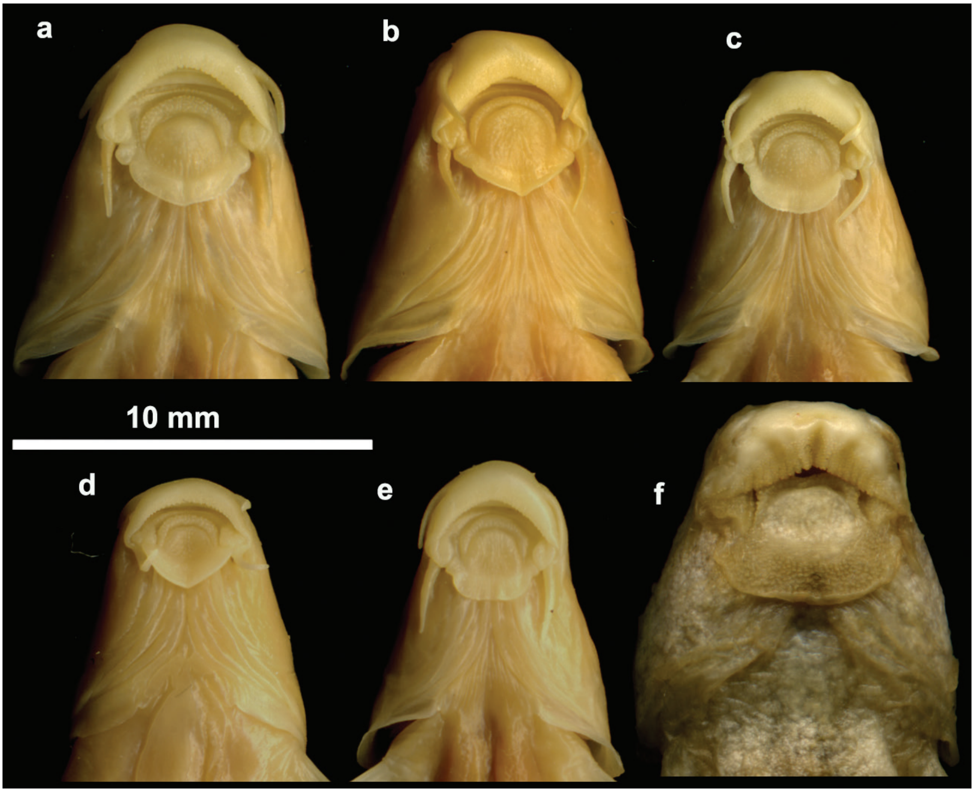

Figure 11. *Garra tashanensis*, head, ventral view: F73, SL 34.8 mm (**a**); F74, SL 40.1 mm (**b**); F75, SL 34.6 mm (**c**), specimen A, SL 33.9 mm (**d**); specimen B, SL 33.1 mm (**e**); and F45, SL 42.2 mm (**f**). Tang-e-Ban Spring, 20.04.2022 (**a–c**) and Tashan Cave (type-locality), 17.03.2018 (**d**).

Comparison between Tashan and Tang-e-Ban samples

As the lengths of two specimens in the Tashan sample were similar to those in most specimens in the Tang-e-Ban sample, we could suppose that differences were not size-dependent. On the other hand, the length of the smaller examined Tashan specimens (SL 22.5–26 mm) corresponds to the size of the holotype and paratypes of the species (SL 22–27 mm) (Mousavi-Sabet et al. 2016). In general, our study was consistent with the original description (Mousavi-Sabet et al. 2016: 136–139), except for the presence of a lateral line (7 pores) in the longer specimen (SL 42.2 mm). Hence, the absence of the lateral line cannot be considered a diagnostic feature of the species, as has been accepted (Zamani Faradonde et al. 2020a, b; Zamani Faradonde and Keyvani 2021) since the description of the species.

A comparison of the samples examined in the present study revealed some clear differences between them. Among the morphometric parameters, the most significant differences (results of the statistical analyses are presented below) are found in the following relative measurements (Table 4) (Tang-e-Ban vs. Tashan): maximum body depth (18–22% SL vs. 24); depth of caudal peduncle (55–69% of caudal peduncle length vs. 80); caudal-peduncle length (18–21% SL vs. 15); body width (14–17% SL vs. 20); pectoral-fin length (17–19% SL vs. 21); head length (25–26% SL vs. 28); head depth at nape (54–60% HL vs. 65); anus – anal-fin origin distance (23–33% pelvic – anal-fin origin distance vs. 19); mouth width (37–45% HL vs. 51); disc width (30–35% HL vs. 44); disc length (91–99% disc width vs. 71); and pulvinus width (25–33% HL vs. 14).

Among the examined morphometric characters, the most prominent differences are commonly 2 unbranched dorsal-fin rays in the Tang-e-Ban sample (vs. 3 in the Tashan sample); commonly 11–12 branched pectoral-fin rays (vs. 13–14); 11–13 predorsal abdominal vertebrae (vs. 10); commonly 9–10 vertebrae between first pterygiophores of dorsal and anal fins (vs. 10–11); arched mouth cleft (vs. straitened), and, the most striking difference, well-developed lateral-line, with 10–34 pored scales imbedded into skin or externally visible (vs. up to maximum of 7 pores without visible scales).

The Tang-e-Ban and Tashan disc-bearing *G. tashanensis* samples are clustered in distinct groups in the CA (Fig. 7a). The PCA implemented using a combination of morphometric and meristic characters (Fig. 8a) show that the F65 specimen is clearly distant from the Tang-e-Ban. Interestingly, in the CA, the hypogean *G. rufa* sample was clustered together with the syntopic *G. tashanensis*, but not with epigean *G. rufa* occurring nearby. When only meristic characters were used, then the two groups were much closer in the PCA (Fig. 7b) and, in CA (Fig. 8b), even clustered together with one specimen of epigean *G. rufa* and one specimen of Tang-e-Ban *G. tashanensis.* In general, the examined specimens of disc-bearing *G. tashanensis* from Tang-e-Ban Spring are very morphologically heterogenous.

Discussion

In this study we reported a cave form of *G. rufa* currently known only from Tang-e-Ban spring (Figs 1, 2), thus, placing this population of the species in this karstic spring into the category of stygobionts. The other species that inhabits Tang-e-Ban spring is genetically clustered with stygobiotic *G. tashanensis*, a species previously known only from Tashan Cave. Our study thus adds a new form of stygobiont fish to complement the taxonomic understanding of the subterranean diversity of Iran. In total, five cave fish species of two genera from two families are now reported from Iran: four species of the genus *Garra* occur in groundwaters of Zagros Mountains, and *Eidinemacheilus smithi* is present in sympatry with two *Garra* species (*Garra lorestanensis* and *G. typhlops*) in Loven cave and Tuveh spring (which are 30 km apart) (Mousavi-Sabet et al. 2016; Malek-Hosseini and Zamani 2017; Malek-Hosseini et al. 2022).

The morphological analysis revealed that, although barcoded as *G. rufa* and *G. tashanensis*, the forms of these species inhabiting Tang-e-Ban significantly differ from the paired forms from the geographically very close Maroon River and Tashan Cave, respectively. As described above, the hypogean *G. rufa* from the Tang-e-Ban spring differs from the conspecific epigean sample from the Maroon River by a variable level of loss of scales (except for the lateral line, which is complete); a continuum of eye reduction (up to a complete loss of externally visible eye structures); 7½ branched dorsal-fin, and a more anterior position of the anal fin relative to the pelvic fin, and a shorter distance between origins of the dorsal and anal fins (expressed both in external measurements and vertebral counts). The Tang-e-Ban *G. tashanensis* mostly differs from the type-bearing phenotype from Tashan cave in the body shape expressed in many relative measurements, commonly 2 unbranched dorsal-fins; a narrower disc; an arched mouth; and, the most striking difference, a well-developed lateral-line, with 10–34 pored scales imbedded into skin or externally visible (vs. up to maximum of 7 pores without visible scales). Morphological diversity of *G. tashanensis* is even higher as there is one more phenotype, a discless form, that occurs in syntopy with the disc-bearing phenotype in Tashan Cave (Hashemzadeh Segherloo et al. 2022; our data).

To distinguish between species, both morphological and genetic criteria should ideally be considered (Bond et al. 2022). Here, the K2P pairwise distance data between population of *Garra rufa* from Tang-e-Ban Spring and other localities was not sufficient to warrant description of a new species, despite the fact that different divergence rates have been applied as decisive criteria for new species (Ward et al. 2009; Esmaeili et al. 2016). This was similar for *G. tashanensis* from Tang-e-Ban Spring and Tashan Cave. These populations of *Garra* from Tang-e-Ban Spring, Tashan cave, Sarjowahar Spring, Maroon River, and also what we obtained from the literature, were conspecific with *G. rufa* and *G. tashanensis*, although some morphological differences and also genetic distances from 0.15% to 1.09% in barcoding COI gene were detected. We consider these as morphotypes of the same species resulting from environmental differences and/or prolonged isolation.

Our phylogenetic results were congruent with previous studies by Esmaeili et al. 2016, Kirchner et al. 2020 and Mousavi-Sabet et al. 2016. Phylogenetic studies based on a fragment of the COI gene (Kirchner et al. 2020) have revealed a close relationship of *Garra rufa* with a number of species, *G. persica* (Iran), *G. widdowsoni* (cave species from Iraq), *G. mondica* Sayyadzadeh (Iran), *G. amirhosseini*, *G. elegans* (Iran), *G. barreimiae* (UAE), *G. ghorensis* (Jordan), *G. jordanica* (Syria and Jordan), *G. typhlops*, *G. gymnothorax*, *G. lorestanensis* (Iran), and *G. longipinnis* (Oman). The sister group of this large clade is *G. tashanensis*. More markers from the abovementioned taxa and also other Iranian *Garra* species must be included in such studies to gain wider insights into the phylogenetic relationships of this group.

More studies are needed to examine the possibility of hybridisation between the two cave species occurring in Tang-e-Ban spring. Another question that will require genetic analyses is whether gene flow occurs between the epigean and hypogean morphotypes of *G. rufa*. We cannot exclude the possibility of existence of an epigean form of *G. tashanensis* in this area. A connection of Tashan cave with the Tang-e-Ban spring is also suggested by the occurrence of *G. tashanensis* in these two localities and may indicate the presence of an important aquifer in the Tashan area, consistent with unofficial reports by locals of “reddish-pinkish fishes” inhabiting other parts of the Tashan region.

Cave populations may remain interfertile with the ancestral surface form and, therefore, may not evolve into separate, reproductively isolated species, or subsequently they may split from the original epigean species following long isolation. Isolation of cave populations of *Garra* fishes can be quite old: for instance, the Somalian cave-dwelling species *Garra andruzzii* (Vinciguerra, 1924) became isolated about 5.3 Mya (at least 2.5 and at most 9.0 Mya; Calderoni et al. 2016), in contrast to *Astyanax mexicanus* where at least five independent events have led to cave-dwelling populations (still completely interfertile with the ancestral surface form) over the past 1–2 Mya (Bradic et al. 2012; Gross 2012).

As a result of old isolation, a diversity of situations is presently observed in *Garra*: there are some exclusively hypogean species such as *G. widdowsoni*, *G. lorestanensis*, and *G. typhlops* for which ancestral epigean forms remain unknown, whereas other species such as *G. longipinnis* and *G. barreimiae* include populations with both epigean and hypogean phenotypes (Banister 1984; Khalaf 2009; Kruckenhauser et al. 2011; Kirchner et al. 2017; Pichler et al. 2018; Kirchner et al. 2020; Freyhof et al. 2020; Sayyadzadeh et al. 2023). Further studies will be required to test the hypothesis that the latter situation corresponds to more recent cave colonisation events than the former.

Tang-e-Ban is a seasonal spring that flows during the period of February to June in highly rainy years. The spring is completely dry during the whole year with low precipitation and also from July until February-March in high-precipitation years. Several outflows for this spring exist, and it is not clear from which part fishes wash out. There are several springs close to Tang-e-Ban whose waters join together through agriculture lands with irrigation. Fish specimens either die in the spring or enter streams and the river. The whole area of Tashan will require comprehensive fieldwork and study to elucidate these mechanisms.

Our discovery of cave fishes in the Tashan area, as well as the presence of other troglobiotic/stygobiotic animals in Tashan cave (including a gastropod and an isopod) (Khalaji-Pirbalouty et al. 2018; Fatemi et al. 2019) reveal that this area should be considered a unique habitat that is worthy of urgent conservation, as numerous threats such as pollutants from human waste, water extraction, fish collection by locals, and uncontrolled human visits are putting the conservation of this unique habitat in danger.

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Supplementary material 1

GenBank accession numbers for COI (codes in bold are our original sequences)

Authors: Mohammad Javad Malek-Hosseini, Jean-François Flot, Yaser Fatemi, Hamid Baboli Moakher, Matjaž Kuntner, Oleg A. Diripasko, Dušan Jelić, Nina G. Bogutskaya

Data type: pdf

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Supplementary material 2

Alignment S1

Authors: Mohammad Javad Malek-Hosseini, Jean-François Flot, Yaser Fatemi, Hamid Baboli Moakher, Matjaž Kuntner, Oleg A. Diripasko, Dušan Jelić, Nina G. Bogutskaya

Data type: fas

Explanation note: Fasta file of COI sequences used in our analyses.

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Aquatic microdiversity from urban cenotes in Cancun, Quintana Roo, Mexico

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Abstract

The microdiversity of cenotes in the Yucatan Peninsula, Mexico has been little studied, with the phytoplankton and protists being the most representative species. However, all previous studies have been focused on cenotes associated with touristic activities, leaving a gap in the understanding of cenotes located within urban areas. The present study is dedicated to the identification of phytoplankton and protists in the cenotes of Cancun, Quintana Roo, Mexico. We conducted our research in four urban cenotes, collecting samples using a 150 µm plankton net, filtering them with a 45 µm membrane, and examining them under optical microscopy. Subsequently, we calculated the abundance, richness, and completeness of the samples. Our findings revealed a total of 6 phyla, 4 subphyla, 10 classes, 8 subclasses, 15 orders, 15 families, 18 genus, and 17 species and 4 species indeterminata in the cenotes of Cancun, Quintana Roo, Mexico. Among these, there were 8 species of phytoplankton and 1 species indeterminata, while 9 species of protists and 3 species indeterminata. These results highlight the remarkable species richness and the complex structure and composition of urban cenotes, suggesting that some species may be unique to this particular ecosystem. Currently, there is limited knowledge regarding the behavior of these aquifers (urban cenotes), and a comprehensive inventory or characterization of their microdiversity is lacking. Such information could be instrumental in the management, conservation, and sustainable use of these valuable aquifers.

Keywords
Microdiversity, phytoplankton, protists, urban cenotes

Introduction

Cancun is located in the northern part of the Yucatan Peninsula within the state of Quintana Roo, Mexico. The karst relief of the Yucatan Peninsula is formed by depressions, sinkholes, and caverns. Occasionally, some of these caverns collapse, producing “cenotes,” a word of Mayan origin (“*ts’ ono ‘ot or*” “*d’zonot*”) that means “cave with a deep pool”, referring to any location with accessible groundwater (Back 1985). Rainwater infiltrates and accumulates in the subsoil of the karst, creating a column of fresh water that rests atop a denser saline water mass resulting from natural seawater intrusion. The contact between these two water masses, the freshwater and marine, forms a mixing zone known as a halocline. This freshwater mass constitutes the only source of freshwater within the Yucatan Peninsula. The formation of cenotes is a consequence of the karst process occurring within the peninsula, resulting from a complex sequence of events. It starts from a flooded cave, and a grotto or a pitcher-type cenote can be formed by the collapse or partial collapse of the roof. Subsequently, the complete collapse of the ceiling results in the formation of a cylindrical cenote. From the cylindrical cenote, an aguada-type cenote may evolve due to sedimentation and the gradual subsidence of the adjacent area (Fig. 1) (Gaona-Vizcayno et al. 1980; Schmitter-Soto et al. 2002a).

The karst relief of the Yucatan Peninsula provides the environmental conditions, and the aquifer’s unique characteristics contribute to the formation of a distinctive ecosystem primarily reliant on microbiome activity (Back 1995; Batllori-Sampedro et al. 2012). Microbiological components within cenotes are throughout the water

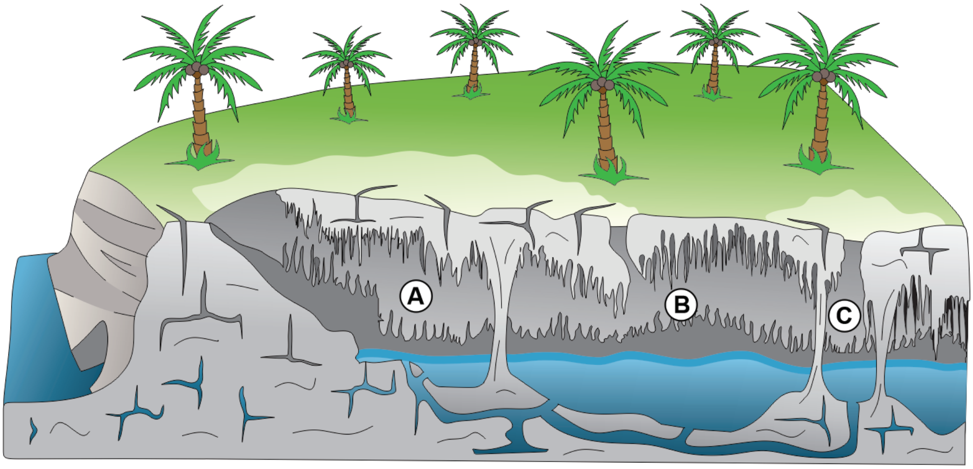

Figure 1. Types of cenotes in the Yucatan Peninsula, Mexico **A** cavern **B** grotto or pitcher-type, and **C** cylindrical cenote.

column, predominantly near the surface. However, the biodiversity of phytoplankton and protists in both fresh and marine waters in the Caribbean region of Mexico has remained unexplored. Indeed, there is a substantial knowledge gap concerning these aquatic systems and their phytoplankton and protists communities (Álvarez-Cadena et al. 2007). Only a few studies have delved into the biodiversity within cenotes, particularly focusing on phytoplankton or cyanobacteria (Arana-Ravell et al. 2019; Moore et al. 2019), while others have explored microbial diversity in cenote sediments and the water columns (Schmitter-Soto et al. 2002b; Suárez-Moo et al. 2022). These investigations have identified species associated with various phyla, including Bacillariophyta, Cryptophyta, Chlorophyta, Chrysophyta, Euglenophyta, Pyrrophyta, Xanthopyta, Dinophyta and the most dominant Cyanobacteria. Some of the orders presented in the cenotes are Synechococcales, Chroococcales, Oscillatoriales, Nostocales, Spirulinales, Pleuroscapsales and Chroococcidiopsidales. Representative genus such as *Flavobacterium*, *Prochlorococcus*, *Brevundimonas*, *Rhodobacter*, *Novosphingobium*, *Desulfobacterium*, *Acinetobacter*, *Pseudomonas*, *Chroococcus*, *Tetrastrum*, *Cryptomonas*, *Encyonopsis*, *Pseudanabaena*, *Aphanocapsa*, *Epigloeosphaera*, *Monoraphidium*, *Brachysira*, *Encyonopsis*, among others, have been commonly observed in these cenote ecosystems.

Each study performed in the cenotes of Yucatan Peninsula, Mexico, with a focus on microbial diversity, has consistently revealed a significant number of species within the sampled sites, along with the variations in the abundance and presence of species between the rainy and dry seasons. However, it is essential to note that all of these prior investigations were conducted in cenotes associated with tourist activities, such as swimming or diving, or in cenotes situated in rural areas or dense jungles. The present study is focused on cenotes located within urban settings, such as Cancun, Quintana Roo, Mexico. These urban cenotes are surrounded by residential units and roadways, presenting a distinctive ecological context.

Urban cenotes, unlike their counterparts in more pristine environments, lack a specific designated purpose. Some of these cenotes are situated within public parks, nestled between bustling avenues, or located on private properties. Only a handful of them are suitable for swimming, while many suffer from issues like litter, discarded tires, and even electrical waste contamination. Despite these significant challenges, research on urban remains limited. Microbial diversity within these cenotes holds particular importance, as it serves as a natural bioindicator of eutrophication and environmental impact. Cell abundance in these ecosystems is influenced by various factors, including biological factors, nutrient levels, organic matter content, pH, mineralization, and more (Darley 1987; Livingston 2001). However, due to the absence of comprehensive data, distinguish between endemic and foreign species within these ecosystems remains a challenge. Furthermore, identifying which species can effectively serve as bioindicators of environmental impact or eutrophication is currently beyond our reach. This study aims to address these knowledge gaps by exploring species richness, community structure, and composition in urban cenotes, with a particular focus on phytoplankton and protists. The data generated through this research can play a crucial role in guiding the management, conservation, and sustainable use of these unique and vulnerable ecosystems.

Methods

Study area

Cancun is located within the municipality of Benito Juárez in the state of Quintana Roo, Mexico (Fig. 2A). Its precise geographical coordinates are 21°09.41'N, 86°49.29'W. The topography of the region is notably flat, with elevations seldom exceeding 10 m above sea level. The predominant vegetation type is a subdeciduous tropical forest, and the area boast an average annual temperature of 25 °C (Rzedowski 2006). For the study we collected samples from four cenotes located within the city of Cancun (Fig. 2B), designated as C1 (a mix between cavern and aguada-type cenote), C2 (a grotto or a pitcher-type cenote), C3 (an aguada-type cenote), and C4 (an aguada-type cenote), corresponding to cenotes 1–4, respectively (Suppl. material 1).

Sample collection

Sample collection was conducted during two distinct seasons of the year: the rainy season, spanning from September to December, characterized by higher rainfall rates; and the dry season, occurring between April and July, marked by reduced precipitation, elevated temperatures, and increased evaporation. The sampling campaign commenced in September 2017 and concluded in July 2018. At each sampling station, two separate water samples were obtained. Each sample consisted of 10 liters of water, which underwent filtration using a 150 µm plankton net (Aquatic Biotechnology, 40cmØx233cmL CP3-110). Subsequently, the samples were suspended in 300 mL of water sourced from the same cenote and stored in sterile 500 ml screw-cap bottles at room temperature. One of the pared samples was fixed using a 4% Lugol solution, while the other was analyzed in its fresh state, following a modified method by Delgado and Sánchez (2006).

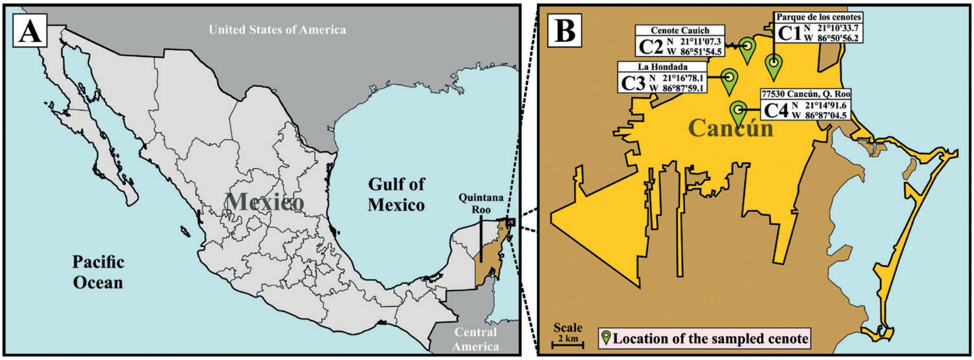

Figure 2. Sampled cenotes in Cancun, Quintana Roo, Mexico **A** Mexico map highlighting Quintana Roo in light brown, with a small box indicating the location of Cancun **B** Cancun map in yellow with green location markers representing the global coordinates of the sampled cenotes: C1 (Parque de los cenotes), C2 (Cenote Cauich), C3 (La Hondada), and C4 (77530 Cancun, Quintana Roo).

Sample processing, taxonomic identification and quantification method

After collection, the samples were stored at room temperature for a maximum of 24 hours. Subsequently, they were filtered through a sterile test tube using a Buchner funnel containing a 45 µm membrane (Millipore). The material trapped on the membrane was resuspended in 1 ml of distilled water for immediate observation as soon as possible. For analysis, taxonomic identification, and documentation, the samples were examined under an optical microscope (Motic and Labomed) with the 10×, 40×, and 100× objectives. Images were recorded using a Sony camera. Each microorganism was identified using established taxonomic keys (Luna-Pabello 2006; Barrios-Barcia and Puig-Infante 2012; Sigala-Regalado et al. 2016; Quiroz-González and Rivas-Acuña 2017; Guiry and Guiry 2020; WoRMS 2020; Siemensma 2023).

To determine the number or organisms, the following equation was applied:

$$\frac{N}{L} = \frac{((C)(v1))}{((v2)(v3))}$$

where N is the total number of organisms, C denotes the number of organisms that were quantified, v1 signifies the volume that was concentrated, v2 indicates the volume used for counting, and v3 represents the volume that was sampled (Gómez-Márquez et al. 2013). The results are expressed as the number of organisms per liter (L) or milliliter (mL).

Completeness analysis

To assess the completeness of the species inventory for each cenote, we employed species accumulation curves as proposed by Colwell and Coddington (1994). These curves were calculated using ACE Mean and Chao 1 Mean estimators, recognized as the most reliable methods for assessing large communities. Additionally, estimations were made for species represented by one (singletons) or two (doubletons) individuals within the samples, following the methodology mentioned by Colwell and Coddington (1994). The underlying assumption of these estimators is that as the number of samples increases and the accumulation curves approach intersection, the inventories approach completeness (Jiménez-Valverde and Hortal 2003). All species accumulation curves were generated using the ESTIMATES ver. 9.1.0 program (Colwell and Coddington 1994).

Structure and species diversity

Rank-abundance curves were employed to evaluate the structure and composition of species within each community, facilitating the identification of dominant and rare species in each environment, following the methodology outlined by (Magurran 1998).

To quantify the diversity within each community, we utilized the Shannon-Wiener index, considering the effective number of species (Jost 2006). The true diversity value was expressed as ID = exp(H'), where ID represents the true diversity for each community, and exp(H') signifies the exponential of the Shannon index (Jost 2006).

Beta diversity (β)

To obtain the degree of similarity between species and types, we used a dendrogram (cluster) from a cluster analysis by Ward's method, which indicates, at the same time, the correlation coefficient between each type of environment (Magurran 2004).

The results obtained from the true diversity analysis allowed for comparisons of the dissimilarity in diversity between communities and the magnitude (percentage) that sets them apart from each other. To calculate the percentage of diversity dissimilarity between communities, we applied the formula (DB×100)/DA, where DA represents the diversity of community A, and DB represents the diversity of community B (Moreno 2001).

Results

We registered a total of 6 phyla, 4 subphyla,10 classes, 8 subclass, 15 orders, 15 families,18 genus, 17 species and 4 species indeterminata (Table 1). To among the four cenotes studied in Cancun, we identified 17 species. Of this species total, 38.09% (8 species) were phytoplankton, while 52.94% (9 species) were protists, including 4 species indeterminata (Fig. 3). This study contributed 11 new records for the Yucatan Peninsula, including the following species: *Euglena mutabilis*, *Lepocinclis acus*, *Phacus orbicularis*, *Coscinodiscus radiatus*, *Oscillatoria limosa*, *Arcella gibbosa*, *Amoeba radiosa* and *Mayorella vespertilioides* and the genus *Vorticella* sp., *Aspidisca* sp. and *Coleps* sp. (Table 1).

We found high completeness percentages in the inventories of all sampled cenotes, with cenote C3 standing out for achieving 100% completeness (Fig. 4). During the rainy season, we identified 12 species in C1; eight species in C3 and seven species in C4. However, no species were identified in cenote C2 cenote during this season. In contrast, during the dry season, the highest species diversity was observed in C1, where we detected four species. C2 and C3 each had two species, while C4 we had one species (Fig. 5).

The communities' structures exhibited moderate equality in C3 and C2, while a particularly high degree of equality was observed in C1 compared to the other sites. In contrast, C4 displayed relatively low equality. Overall, no dominant species were observed except for *Euglena mutabilis*, *Radiocystis geminata*, and *Aspidisca* sp., which were prominently represented in C1 and C2. However, C3 and C4 did not exhibit any dominant species (Fig. 6).

The cenote exhibiting the highest diversity, as indicated by the Shannon-Wiener index and beta diversity analysis, is C1, with a diversity index value of 2.3, followed by C3 with an index of 1.7. In contrast, the less diverse sites were C4 with a value of 1.6 and C2 with a value of 0.6. When evaluating diversity in relation to seasonality, we observed greater diversity during the rainy season, with an index of 3.2. In contrast, during the dry season, the diversity index was generally lower, with a value of 2.5 across all sites. The beta diversity analysis revealed low species similarity between cenotes, suggesting that each cenote harbors exclusive species (Fig. 7).

Table 1. List of species recorded in the study area. The species indicated with an asterisk are the new records for the Yucatan Peninsula.

Phylum Euglenophyta
Subphylum: Euglenoida
Class Euglenophyceae
Subclass Euglenophycidae
Order Euglenales
Family Euglenaceae
Genus *Euglena*
**Euglena mutabilis* F. Schmitz, 1884
Euglena texta (Dujardin) Hübner, 1886

Family Phacaceae
Genus *Lepocinclis*
**Lepocinclis acus* (O.F. Müller) B. Marin and Melkonian 2003

Genus *Phacus* Dujardin, 1841
* *Phacus orbicularis* Hübner, 1886
Phacus longicauda (Ehrenberg) Dujardin 1841

Phylum Heterokontophyta
Subphylum Ochrophytina
Class Chrysophyceae
Order Chromulinales
Family Dinobryaceae
Genus *Dinobryon*
Dinobryon sertularia Ehrenberg, 1834

Phylum Bacillariophyta
Subphylum Bacillariophytina
Class Bacillariophyceae
Subclass Coscinodiscophycidae
Order Coscinodiscales
Family Coscinodiscaceae
Genus *Coscinodiscus*
**Coscinodiscus radiatus* Ehrenberg, 1840

Subclass Bacillariophycidae
Order Thalassiophysales
Family Catenulaceae
Genus *Amphora*
Amphora ocellata Ehrenberg, 1838

Subclass Coscinodiscophycidae
Order Thalassiosirales
Family Stephanodiscaceae
Genus *Cyclotella*
Cyclotella meneghiniana Kützing, 1844

Phylum Cyanobacteriota
Class Cyanophyceae
Subclass Oscillatoriophycidae
Order Oscillatoriales
Family Oscillatoriaceae
Genus *Oscillatoria*
**Oscillatoria limosa* C. Agardh ex Gomont, 1892

Order Chroococcales
Family Microcystaceae
Genus *Radiocystis*
  Radiocystis geminata Skuja, 1948

Genus *Merismopedia*
  Merismopedia angularis R.H. Thompson, 1939

Order Pseudanabaenales
Family Pseudanabaenaceae
Genus *Pseudanabaena*
  Pseudanabaena mucicola (Naumann & Huber-Pestalozzi) Schwabe 1964

Phylum Ciliophora
Subphylum Intramacronucleata
Class Spirotrichea
Subclass Hypotrichia
Order Euplotida
Family Aspidiscidae
***Genus** *Aspidisca* Ehrenberg, 1830

Class Oligohymenophorea
Subclass Peritrichia
Order Sessilida
Family Vorticellidae
***Genus** *Vorticella* Linnaeus, 1767

Subclass Peniculia
Order Peniculida
Family Parameciidae
Genus *Paramecium*
  Paramecium aurelia Ehrenberg, 1838

Class Prostomatea
Order Prorodontida
Family Colepidae
***Genus** *Coleps* Nitzsch, 1827

Phylum Amoebozoa
Class Tubulinea
Order Arcellinida
Family Arcellidae
Genus *Arcella*
  **Arcella gibbosa* Penard, 1890

Class Lobosa
Order Amoebida
Family Amoebidae
Genus *Amoeba*
  **Amoeba radiosa* Ehrenberg, 1838

Class Discosea
Order Dermamoebida
Family Mayorellidae
Genus *Mayorella*
  **Mayorella vespertilioides* Page, 1983

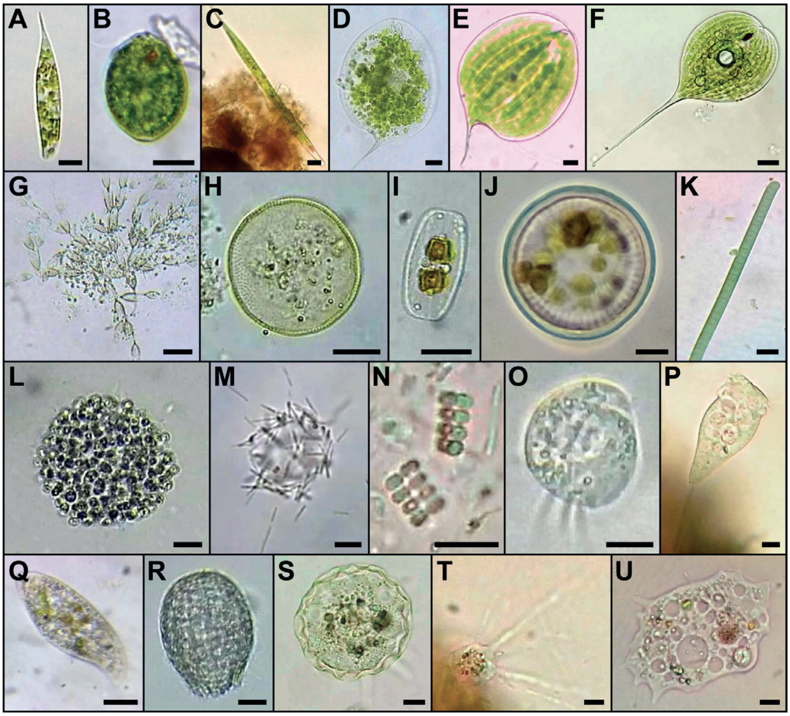

Figure 3. Species found in the sampled cenotes in Cancun, Quintana Roo, Mexico **A** *Euglena mutabilis* **B** *Euglena texta* **C** *Lepocinclis acus* **D** *Phacus* sp. **E** *Phacus orbicularis* **F** *Phacus longicauda* **G** *Dinobryon sertularia* **H** *Coscinodiscus radiatus* **I** *Amphora ocellata* **J** *Cyclotella meneghiniana* **K** *Oscillatoria limosa* **L** *Radiocystis geminata* **M** *Pseudanabaena mucicola* **N** *Merismopedia angularis* **O** *Aspidisca* sp. **P** *Vorticella* sp. **Q** *Paramecium aurelia* **F** *Coleps* sp. **S** *Arcella gibbosa* **T** *Amoeba radiosa* **U** *Mayorella vespertilioides*. Scale bars: 10 µm.

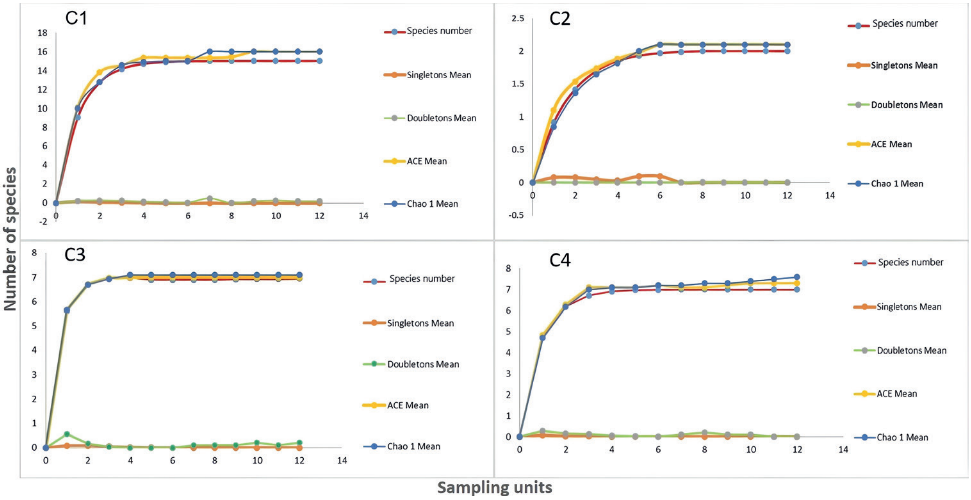

Figure 4. Completeness analysis of species inventories in analyzed cenotes using species accumulation curves and estimators.

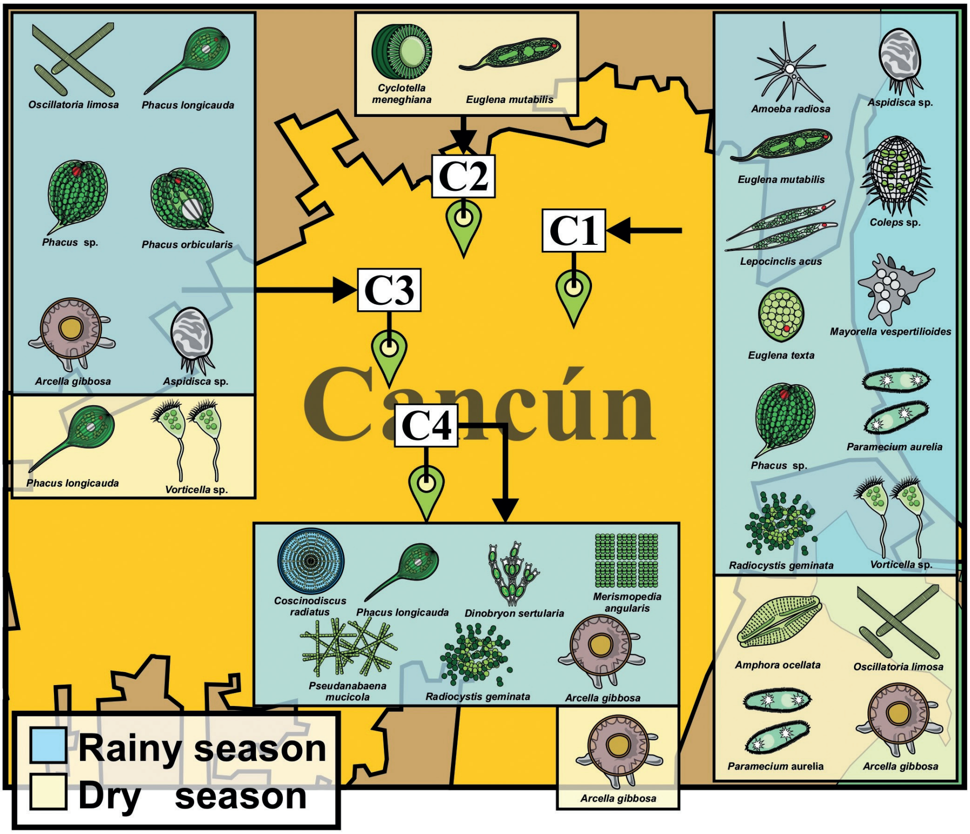

Figure 5. Sampled cenotes in Cancun, Quintana Roo, Mexico for rainy and dry seasons. The city of Cancun is highlighted in yellow. Green location markers show the sampled cenotes: C1 (Parque de los cenotes), C2 (Cenote Cauich), C3 (La Hondada), and C4 (77530 Cancun, Quintana Roo). The blue rectangles show organisms sampled in the rainy season and the light-yellow rectangles show organisms sampled in the dry season.

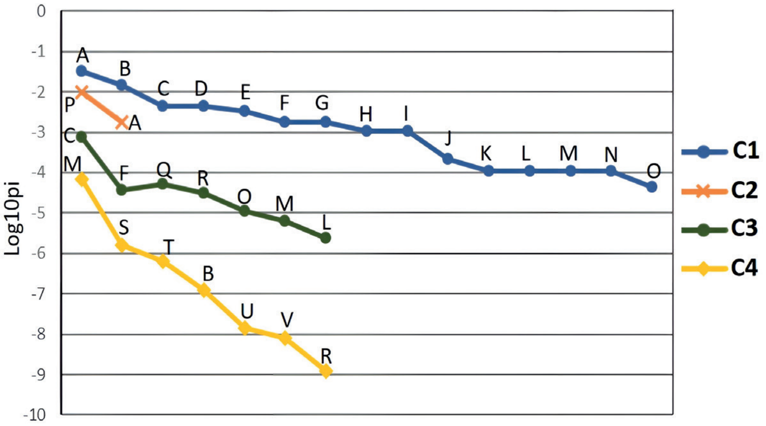

Figure 6. Rank-abundance curves of phytoplankton and protists communities in the 4 sampled urban cenotes in Cancun. C1 corresponds to the abundance found in cenote 1, C2 shows the abundance found in cenote 2, C3 indicates the abundance found in cenote 3, and C4 shows abundance found in cenote 4.

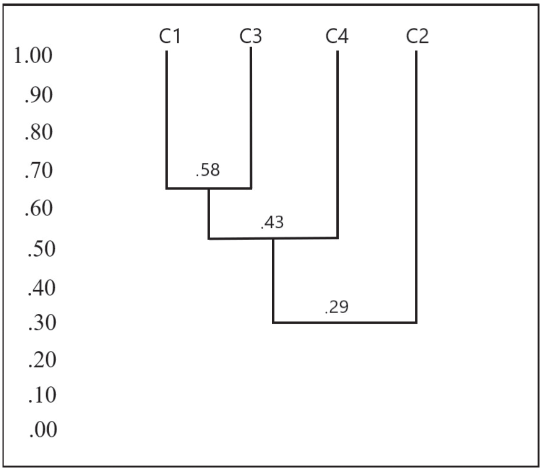

Figure 7. Cluster analysis for each cenote and between cenotes. The analysis shows the diversity similarity found in each cenote and between the different types of habitats analyzed in each urban cenote.

Discussion

The loss of tropical biodiversity has become a growing concern due to the rapidly expanding human population and the increasing demand for resources such as land and water for various habitats (Edwards et al. 2019). Preserving these delicate ecosystems necessitates studies that underscore the urgency of their conservation efforts. For instance, the biodiversity of the coastal ecosystems along México's Gulf and Atlantic coasts faces threats from various anthropic activities. In the Mexican Caribbean, the recent surge in human population has resulted in escalated environmental impacts on both freshwater and marine environments (Guerra-Castro et al. 2020). Despite these challenges, the biodiversity of freshwater and marine ecosystems in the Mexican Caribbean remains poorly studied, resulting in a significant knowledge gap regarding these aquatic systems and their phytoplankton and protists communities. Cenotes, as heterotrophic systems, play a unique role due to the microorganisms' ability to balance the microecosystem, giving rise to a distinct cenote ecosystem (Schmitter-Soto et al. 2002a). While studies have focused on the taxonomic composition of phytoplankton in coastal lagoons in the Yucatan Peninsula (Herrera-Silveira et al. 1999; Nava-Ruiz and Valadez 2012; Valadez et al. 2013), there is a notable absence of references regarding the structure of phytoplankton and protists communities in urban cenotes within the Mexican Caribbean. This study illuminates the richness in the structure and composition of species within cenotes associated with urban regions, which today dominate the landscape. These cenotes hold ecological significance, and their diversity serves as a crucial indicator of ecosystem health.

While the sampling completeness in each of the cenotes is relatively high, hovering around 85%, it is essential to acknowledge that achieving comprehensive representation of microbial species in any given environment is a formidable challenge. Studies aiming to assess species diversity strive to gain a holistic understanding of a site's diversity, but achieving complete representation is often an elusive goal (Hortal et al. 2006), as demonstrated in our study. Several factors contribute to the completeness of the inventories, including sampling bias, methodologies employed for sampling (such as trapping techniques), the timing of sampling, fluctuations in environmental conditions, and the structural complexity of the ecosystem under investigation (Hortal et al. 2006). Nonetheless, it's important to note that a representative sample obtained through systematic sampling can still provide a valuable reflection of a site's diversity (Moreno et al. 2011). Interestingly, our study revealed a higher abundance of protists compared to phytoplankton organisms during the rainy season as opposed to the dry season (Fig. 5). This finding contrasts with the observations of Kouassi et al. (2013) in the Adzopé Reservoir, located in the city of the Adzopé, Ivory Coast, Africa, where they noted greater species abundance during the dry season and fewer species in the rainy season. It's important to note that these differences can be attributed to the distinct characteristics of the two ecosystems. Furthermore, studies conducted in coastal lagoons of the Yucatan Peninsula (Herrera-Silveira et al. 1999; Nava-Ruiz and Valadez 2012) found no significant differences in species richness between seasons but observed variations in dominant species throughout the year. These differences likely stem from the varying tolerance levels and physiological characteristics of individual organisms, as well as the unique environmental conditions in each site.

We identified four phyla of phytoplankton, with five species belonging to Euglenophyta and one species indeterminate, one species to Heterokontophyta, three species to Bacillariophyta and four species to Cyanobacteriota. Notably, Euglenozoa emerged as the most diverse group among them. It's worth highlighting that many of these species represent the first documented records for the Mexican Caribbean (Luna-Pabello 2006; Sigala-Regalado et al. 2016; Quiroz-González and Rivas-Acuña 2017; Guiry and Guiry 2020; WoRMS 2020) (Table 1). Seasonal precipitation has been a crucial factor correlated with an increase in phytoplankton biomass in various aquatic environments (Okoth et al. 2009). This phenomenon has significantly contributed to the heightened production and diversity of phytoplankton and protists species during the rainy season, a trend not previously reported for cenotes in the northern Yucatan Peninsula. Furthermore, Troccoli et al. (2004) observed a relationship between hydrographic variables and phytoplankton blooms in coastal areas along the beaches of Campeche, Yucatan, and Quintana Roo. Their analysis of the three coastal zones suggests that the differences in hydrology and biology between Campeche and Yucatan/Quintana Roo were attributed to marine currents. In cenotes, underground currents play a significant role, so it's crucial to consider variables such as nutrient concentrations, temperature, and various physical parameters as potential drivers of biodiversity.

In our study, a higher density of protists was observed in sampled cenotes compared to phytoplankton. We identified two phyla: Ciliophora with one species and three species indeterminata and Amoebozoa with three organisms. Remarkably, all species, except for *P. aurelia*, represented the first record instances for the Mexican Caribbean region, contributing to an increase in species diversity in the northern Yucatan Peninsula. Furthermore, we observed a higher density of protists during the rainy season, particularly in cenote C1. This finding aligns with the observations of Sigala-Regalado et al. (2011), who emphasized the importance of protists in ecosystems while nothing their limited study in cave environments. Sigala-Regalado et al. (2011) reported eight species of ciliates, three species of flagellates, and one amoeboid species within cave systems in Queretaro, Mexico, over a year. Five of these species were reported for the first time inside cave systems, and an additional three species are new records for caves. In our report, we identified four species and three species indeterminata of which six representing the first recorded instances for the Mexican Caribbean. This variation suggests that each cenote harbors unique species, contributing to low species similarity between the analyzed sites, as demonstrated in the cluster analysis. This pattern is consistent with the observations made by Sigala-Regalado et al. (2011), who noted that each ecosystem or habitat tends to host distinct species with few shared species. Protists exhibit a wide range of dietary requirements (Pratt and Cairns 1985), and these requirements were met by the different cenotes. Additionally, the broad environmental tolerances of common taxa, such as *Aspidisca* sp., *Vorticella* sp. and *P. aurelia*, suggest that these species could potentially be found in nearly every natural system.

Until now, comprehensive studies evaluating the structure and diversity of communities in urban cenotes have been notably lacking. This represents a significant gap in our understanding, as the mere presence of species does not provide insight into the overall quality and ecological health of aquatic systems. Therefore, this work serves as a crucial foundational step for future research endeavors aimed at assessing the richness, abundance, and structural characteristics of these communities. These organisms, occupying the primary trophic level within the ecosystem, play a fundamental role in shaping the entire food web. Consequently, ongoing, and systematic monitoring efforts are imperative to gauge and ensure the health and sustainability of these vulnerable ecosystems.

Conclusions

We identified eight species of phytoplankton and one species indeterminata while, nine species of protists and three species indeterminata in the cenotes of Cancun, Quintana Roo, Mexico. Some of these species represent new records, underscoring the complexity and diversity of these ecosystems. Given the ecological significance of cenotes and their vital role in the economic sustenance of the region, it is imperative to implement effective management and conservation strategies. This is crucial in order to mitigate the potential polluting factors resulting from current cenotes management practices.

Furthermore, compounding the existing challenges, there is currently a lack of precise knowledge regarding the behavior and dynamics of these urban aquifers. This deficiency in information severely hinders effective management strategies to mitigate potential future negative impacts. There exists a notable gap in studies providing comprehensive inventories and characterization of urban cenotes, which are essential for informed decision-making in their management, conservation, and sustainable utilization. Addressing this issue necessitates the implementation of public policies and actions, coupled with technical and scientific support from hydrological systems. Furthermore, active participation from society is vital to collectively protect and conserve these ecosystems.

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Supplementary material 1

Kinds of cenotes sampled

Authors: Job Alí Díaz-Hernández, Paul Ugalde-Silva, Christian Berriozabal-Islas, Alejandro Novelo, Jaqueline Hernandez-Uc, Abigail Arana-May, Sheila Denisse Pech-Patrón, Iris Aurora Nava-Jiménez, Jessica Borbolla-Vazquez

Data type: tiff

Explanation note: Kinds of cenotes sampled (A) C1 (Parque de los cenotes) a mix between cavern and aguada-type cenote; (B) C2 (Cenote Cauich) a grotto or a pitcher-type cenote; (C) C3 (La Hondada) an aguada-type cenote; and (D) C4 (77530 Cancún, Q. Roo) an aguada-type cenote.

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Assessment of occurrence, diversity, and biomass of macroinvertebrates in Swiss groundwater systems using citizen science data

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Abstract

Groundwater is a vast ecosystem harboring a high diversity of specialized taxa. Despite its diversity, groundwater is a still relatively unexplored and threatened ecosystem. Especially the linkage of groundwater with other ecosystems remains largely unknown from the perspective of groundwater fauna. Here, we used citizen science data to get a first baseline knowledge of the occurrence, diversity, and biomass of major macroinvertebrate groups found in shallow groundwater systems of Switzerland. We investigated all organisms collected from the groundwater in 346 spring catchment boxes of municipal drinking water providers. We morphologically identified the organisms on a broad taxonomic level and estimated their biomass and pigmentation using automated image processing. Crustaceans, particularly *Niphargus* and groundwater isopods, were the most common taxa of obligate groundwater organisms. We also found a surprisingly high number of macroinvertebrates associated with surface and subsurface ecosystems. These taxa might be accidentally entering the groundwater or use it as temporary habitat. In both cases they possibly contribute essential allochthonous energy imports from the surface. We found a positive relationship between the estimated biomass of macroinvertebrates in the samples and the occurrence and abundance of *Niphargus*. Owing to the widespread occurrences of surface and subsurface macroinvertebrates in our groundwater samples, our study provides evidence for common interactions between groundwater, soil, and surface ecosystems.

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* These authors contributed equally to this work.

Keywords
Aquifers, hyporheic, pigmentation, stygofauna, subsidy, Switzerland

Introduction

Groundwater harbors a unique and diverse fauna, yet is still an understudied ecosystem (Mammola et al. 2020). This stygofauna contributes substantially to the functioning of groundwater ecosystems, for example by facilitating the breakdown of particulate organic matter (Boulton et al. 2008; Griebler and Avramov 2015). It consists of obligate groundwater organisms (stygobites), as well as organisms that are occasionally or accidentally entering the groundwater realm (stygophiles and stygoxenes) (Gibert et al. 1994). Many of these organisms are vulnerable to rapid environmental changes, making them essential conservation subjects and potential biological indicators for monitoring groundwater quality (Malard et al. 1996; Griebler et al. 2010; Stein et al. 2010).

While attempts to develop ecological indicators for groundwater monitoring have been made (e.g., Hahn 2006; Steube et al. 2009; Korbel and Hose 2011; Griebler et al. 2014a), there is still a long way to go until this method becomes widely applied (Steube et al. 2009; Griebler et al. 2014a). This also reflects the state of research on groundwater ecosystems, which lags behind that of surface water ecosystems (Danielopol et al. 2003; Griebler et al. 2014b; Borko et al. 2022). One of the main causes is the restricted accessibility to groundwater ecosystems (Gibert and Culver 2009; Griebler et al. 2014b). In addition, ecological links between groundwater and other ecosystems have been understudied, despite groundwater aquifers being inherently linked to surface water and soil through water flows and groundwater recharge (Malard et al. 2023).

Along the hyporheic zone, water, dissolved oxygen, nutrients, organic matter, and organisms are exchanged between surface water and groundwater, creating environmental gradients along this transition area (Boulton et al. 2008). Since aquifers are environments that lack photosynthetic primary production, groundwater fauna largely relies on allochthonous energy sources imported from the surface (e.g., Gibert et al. 1994; Humphreys 2006; Fišer et al. 2014), making it a typical example of resource subsidies and meta-ecosystem dynamics (see Gounand et al. 2018). In specific cases, this is complemented by chemolithoautotrophic primary production within groundwater ecosystems themselves (Hutchins et al. 2016; Herrmann et al. 2020). An essential contributor to the energy flow from the surface to groundwater ecosystems is the total inflow of macroinvertebrate biomass (e.g., Benke and Huryn 2007; Machuca-Sepúlveda et al. 2022; Malard et al. 2023).

Drinking water aquifers and subsequent water collection in spring catchment boxes provide novel yet underexplored access to groundwater systems (Alther et al. 2021; Couton et al. 2023a, 2023b). Due to the shallow depth from which the groundwater is drained from the aquifers into these facilities, they enable the study of groundwater biodiversity and the linkage of groundwater to other ecosystems. In Switzerland, promising monitoring approaches of these spring catchment boxes have been established (e.g., Alther et al. 2021; Couton et al. 2023a). For example, studies by Alther (2020) and Studer et al. (2022) show that a citizen science approach is successful in accessing groundwater ecosystems through spring catchment boxes. However, these studies have focused mainly on groundwater amphipods (*Niphargus* spp.) and specific regions of Switzerland, and therefore, the entirety of groundwater fauna across all of Switzerland and the interactions between groundwater and other ecosystems still remain largely unknown and undocumented.

Here, we shed light on the diversity, occurrence, and biomass of macroinvertebrates found in Swiss groundwater systems and on the linkage to other surface and subsurface ecosystems. We used standardized groundwater samples retrieved from spring catchment boxes by local drinking water providers as part of a systematic Swiss-wide citizen science project. We measured the biomass of macroinvertebrates collected in groundwater to understand ecological processes between groundwater and surface ecosystems and its potential as resource influx. Additionally, we used the pigmentation of the macroinvertebrates as an approximate classification into hypogean and epigean fauna.

Methods

Citizen science sampling procedure

The samples were collected between 2021 and 2022 using a Swiss-wide, systematic citizen science approach. We collaborated with local drinking water providers, who sampled the groundwater flowing into spring catchment boxes (hereafter referred to as spring boxes) with filter nets (similar to Alther et al. 2021 and Studer et al. 2022). First, we used a regular grid to select municipalities and contacted the corresponding drinking water providers to ask for participation in our study. We then sent sampling kits and sampling instructions to all drinking water providers interested in participation. The water providers were instructed to install a filter net (mesh size 0.8 mm, Sefiltec AG, Höri, Switzerland) around pipes that passively drain groundwater from the aquifer to the spring box and to repeatedly collect all organisms washed into the filter net every seven days. All organisms were transferred into sample tubes containing 80% ethanol. The samples and a protocol containing supplementary information such as sampling duration and water discharge rate were then returned to us. After receiving the samples, we separated amphipods from other macroinvertebrates and stored all samples at 4 °C. For the subsequent analysis, we included 1,182 samples from 346 sites across Switzerland, for all of which data on water discharge rate and standardized sampling duration was available.

Taxonomic identification

All macroinvertebrates were identified morphologically using a stereomicroscope (Nikon SMZ1500, 0.75–11.25×). We also identified exuviae and fragments of animals when the number of individuals could be inferred. Based on various identification resources (Zettel 2003; Tachet et al. 2006; Schminke and Gad 2007; Altermatt et al. 2019; Klausnitzer 2019; Zaenker et al. 2020; Walser et al. 2021), specimens belonging to the classes Symphyla, Chilopoda, Gastropoda, and Diplopoda were identified to class level, while Hirudinea, Oligochaeta, Collembola, Acari, and Diplura were identified to subclass level. Specimens of the following taxa were identified to order level: Ephemeroptera (larvae), Plecoptera (larvae), Trichoptera (larvae), Coleoptera (adults and larvae), Hemiptera, Diptera (larvae), Isopoda, Araneae, Opiliones, and Pseudoscorpiones. Formicidae (Hymenoptera) specimens were identified to family level, and Amphipoda specimens to genus level. For the order Isopoda, we differentiated between groundwater Isopoda (Asellidae, cf. *Proasellus,* unpigmented) and remaining Isopoda (pigmented). All other specimens not belonging (or not identifiable) to any of these groups were summarized as "unidentified" or "other adult insects" respectively.

Estimation of biomass and pigmentation

We used automated image processing (ImageJ version 1.53t, Rasband 2014) to identify the silhouette of the organisms within each sample and then calculated the area values and the mean grey values (as an estimate of their pigmentation level) of the respective silhouettes. For each sample, we used the sum of the area values (hereafter referred to as bio-area) as a proxy for biomass, as we found a strong positive correlation between the two in a subset of the data (Suppl. material 1). To create the images, we transferred all macroinvertebrate specimens (including fragments and exuviae) of each sample into a petri dish (d = 8.5 cm) filled with 80% ethanol. We placed the petri dish in a predefined position over a blue, laminated paper that contrasted with both pigmented and unpigmented specimens (Suppl. material 2: fig. S2). We took one picture of each sample with a digital camera (Nikon D5600 with Nikkor 18–55 mm lens) fixed to a camera stand. We measured the area (mm^2) of each organism and quantified its average pixel intensity with batch processing in ImageJ (see Suppl. material 2 for batch processing code and additional information). The CSV files containing the area measurements and the mean grey values of all individuals of a sample were processed in R version 4.1.2 (R Core Team 2021). Measurements for *Niphargus*, *Gammarus*, and oligochaetes were compiled and added separately, as these specimens were previously sorted out for further work (Suppl. material 2).

Statistical analysis

We standardized the bio-area and taxonomic abundances by the sampling duration and the volume of discharged groundwater (retrieved from the sampling protocol filled by the drinking water providers). Therefore, we calculated the bio-area of each site per 100 megaliters of discharged groundwater (1 ML = 1,000,000 liters). For the taxonomic abundances we standardized by 1,000 ML discharged groundwater per site. Based on the mean grey value of all organisms, we additionally split the standardized bio-area of each sample into light-pigmented (mean grey value above 111.86) and dark-pigmented (mean grey value below 111.86) bio-area. The threshold for the categorization was set based on the mean grey values of groundwater Isopoda (Asellidae, cf. *Proasellus*, unpigmented) specimens. All statistical analyses were performed based on the standardized data.

We extracted for each sampling site the aquifer type and tested if diversity, bio-area and pigmentation ratio varied between aquifer types. Therefore, we included the three dominant aquifer types present in Switzerland, namely fissured, karstic, and unconsolidated aquifers. The geodata for the aquifers was retrieved from BAFU (2017). We calculated the following local diversity metrics based on taxonomic orders for each site: richness, Shannon index, and Pielou's evenness. These indices were calculated using the R package vegan (version 2.5–7, Oksanen et al. 2020). We then evaluated whether the diversity metrics, as well as the total standardized bio-area and proportion of light-pigmented bio-area values differed between aquifer types using Kruskal-Wallis rank-sum tests and post-hoc pairwise Wilcoxon rank-sum tests with Bonferroni correction.

To test for a difference between the amounts of light- and dark-pigmented, standardized bio-area per sample, we used a paired Wilcoxon rank-sum test. For samples including both pigmentation categories, we additionally computed Kendall's Tau to assess the correlation between the amounts of light- and dark-pigmented bio-area of the samples. We analyzed the effect of the bio-area on *Niphargus* occurrence with a generalized linear model (GLM), using a binomial error distribution. Twelve sites with a very high amount of bio-area (> 40,000 mm^2 per 100 ML discharged groundwater) had a large impact on the results of the model and were thus removed from the analysis. To analyse the relationship between *Niphargus* abundance and macroinvertebrate bio-area, we compared three different models, that all accounted for zero-inflation, since *Niphargus* was not detected in 66.18% of the sampling sites. We applied two zero-inflated negative binomial (ZINB) models (with and without square-root-transformed response and explanatory variables) and a zero-inflated Poisson (ZIP) model, using the R function "zeroinfl()" from the package pscl (Jackman 2020). The best model was selected based on the dispersion statistic and the Akaike Information Criterion (AIC).

All analyses were performed using RStudio version 2023.03.0+386 on R version 4.1.2 (R Core Team 2021). Figures were produced using the package ggplot2 (Wickham 2016).

Results

Using a citizen science approach, we obtained 1,182 standardized filter net samples collected by municipal drinking water providers from 346 spring boxes (Fig. 1A). For all of these samples, data on water discharge rate and sampling duration was available.

Overall, 404 samples were empty and 778 samples contained a total of 5,578 macroinvertebrate individuals (including fragments and exuviae). Out of those, we identified 5,390 individuals belonging to 9 classes (Insecta, Malacostraca, Diplopoda, Chilopoda, Symphyla, Arachnida, Clitellata, Gastropoda, and Entognatha). 4,408 of those individuals were additionally identified to the order level. The remaining 188 individuals could not be identified.

Plecoptera (larva) and the two stygobiotic taxa *Niphargus* and groundwater Isopoda (Asellidae, cf. *Proasellus*, unpigmented) were the most abundant taxonomic groups. The least abundant taxon was Diplura (Suppl. material 3: fig. S4). We found macroinvertebrates in 271 out of 346 sites (78%) (Fig. 1A). The three taxa found in most spring boxes were *Niphargus* (34% of sites), other adult Insecta (30% of sites), and adult Coleoptera (27% of sites) (Fig. 1B). Groundwater Isopoda (Asellidae, cf. *Proasellus*, unpigmented) were found in 21% of sites (Fig. 1B).

When comparing the local macroinvertebrate diversity (Shannon index) and richness at the order level between aquifer types, a Kruskal-Wallis test revealed a statistically significant difference in local diversity ($X^2_{(2)} = 6.54$, $p = 0.038$) and richness ($X^2_{(2)} = 15.04$, $p < 0.001$) (Fig. 2). Post-hoc pairwise Wilcoxon tests indicated a higher median for diversity in unconsolidated aquifers in comparison to fissured aquifers ($p < 0.05$). For richness, both, unconsolidated ($p < 0.05$) and karstic ($p < 0.05$) aquifers had higher medians in comparison to sites within fissured aquifers. Despite finding some evidence for a difference in Pielou's Evenness ($X^2_{(2)} = 6.02$, $p = 0.049$), none of the pairwise comparisons between aquifer types was significant.

The amount of standardized macroinvertebrate bio-area ranged from 0 to 396,991 mm^2 per 100 ML discharged groundwater. The median was 953 mm^2 per 100 ML discharged groundwater. Using a Kruskal-Wallis test, we did not find any significant difference of the standardized bio-area between different aquifer types ($X^2_{(2)} = 4.56$, $p = 0.10$).

In total, 40% of the standardized macroinvertebrate bio-area sampled across all Switzerland was light-pigmented and 60% dark-pigmented. Based on the 271 sites where any bio-area was obtained, 67% of the sites had dark and light-pigmented bio-area, whereas 20% had dark-pigmented only and 13% light-pigmented bio-area only. The median amount of dark-pigmented standardized bio-area per sample was significantly higher than the median amount of light-pigmented area (paired Wilcoxon test, $p < 0.05$, Fig. 3). For samples including both pigmentation categories, we found a positive correlation between the amounts of standardized light- and dark-pigmented bio-area (Kendall's Tau = 0.48, $p < 0.05$). No significant difference was found when comparing the proportions of light-pigmented bio-area per sample between aquifer types (Kruskal-Wallis test, $X^2_{(2)} = 0.35$, $p = 0.84$).

Based on the standardized data, we compared the occurrence and abundance of *Niphargus* with the bio-area of other macroinvertebrates. There were 75 sites with empty samples (no *Niphargus* and no other macroinvertebrate bio-area) and 105 sites where both *Niphargus* and other macroinvertebrates were obtained. There were 154 sites without *Niphargus* but with other macroinvertebrates (bio-area of other macroinvertebrates > 0) and 12 sites where only *Niphargus* was detected (bio-area of other macroinvertebrates = 0). The binomial GLM showed a tendency for *Niphargus* occurrence to increase with increasing bio-area of other macroinvertebrates (Fig. 4 and Suppl. material 3: table S1). Based on the dispersion statistic and the AIC, we selected the ZINB model with square-root-transformed response and explanatory variables (Suppl.

material 3: table S2). The ZINB regression model showed that *Niphargus* abundance significantly increased with increasing bio-area of other macroinvertebrates (Fig. 5 and Suppl. material 3: table S3).

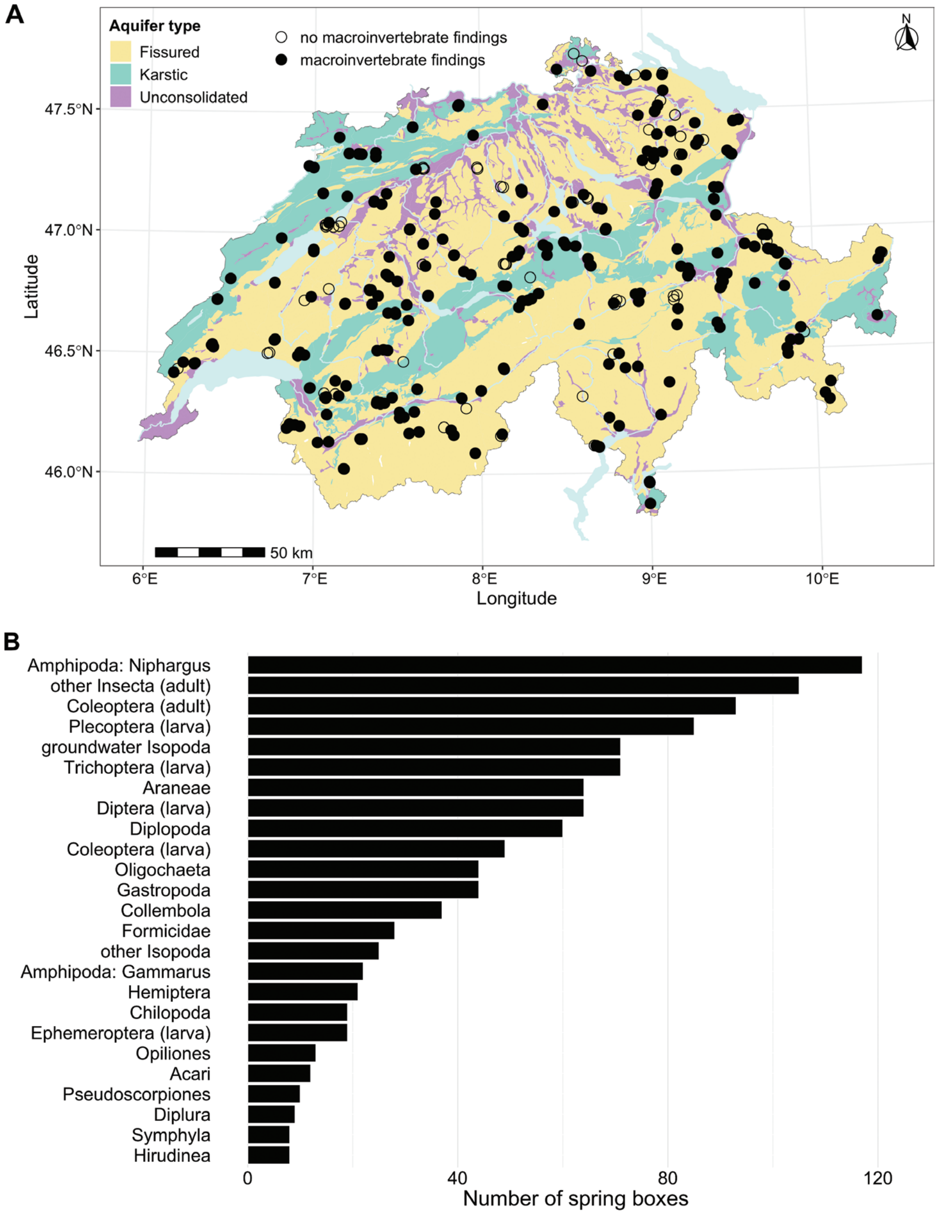

Figure 1. Sampling locations and macroinvertebrate occurrences **A** map of the main aquifer types in Switzerland (BAFU 2017) and the sampling sites (total n = 346). Filled circles indicate sites where macroinvertebrates were found and open circles mark sites where no macroinvertebrates were found. Geodata from the Swiss Federal Office of Topography **B** number of spring boxes (total n = 346) where each major macroinvertebrate group was found.

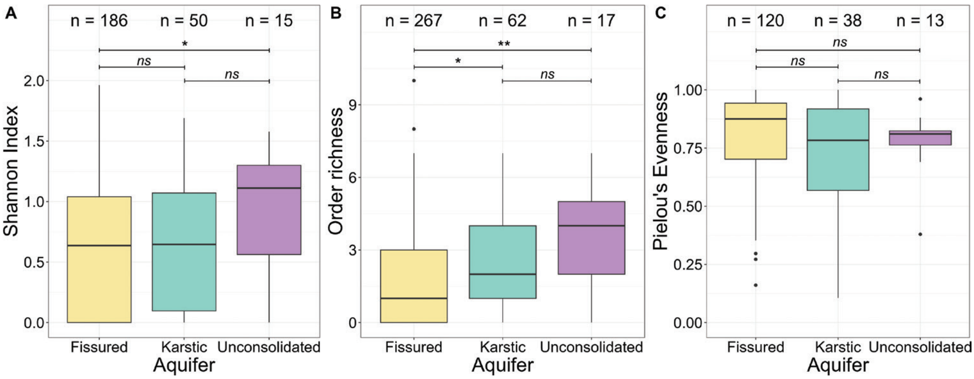

Figure 2. Local macroinvertebrate diversity at the order level per aquifer type **A** shannon Index (sites with at least one order included) **B** order richness (number of orders per site, all sites included), and **C** Pielou's Evenness (sites with at least two orders included). The thick horizontal lines show the median, the interior of each box represents the interquartile range (IQR) and the vertical lines represent minima and maxima, respectively (1.5 * IQR). The number of sites included for each analysis is shown on top of each boxplot, as well as the significance level between groups (pairwise Wilcoxon rank-sum test with Bonferroni correction, *ns* for $p > 0.05$, * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$).

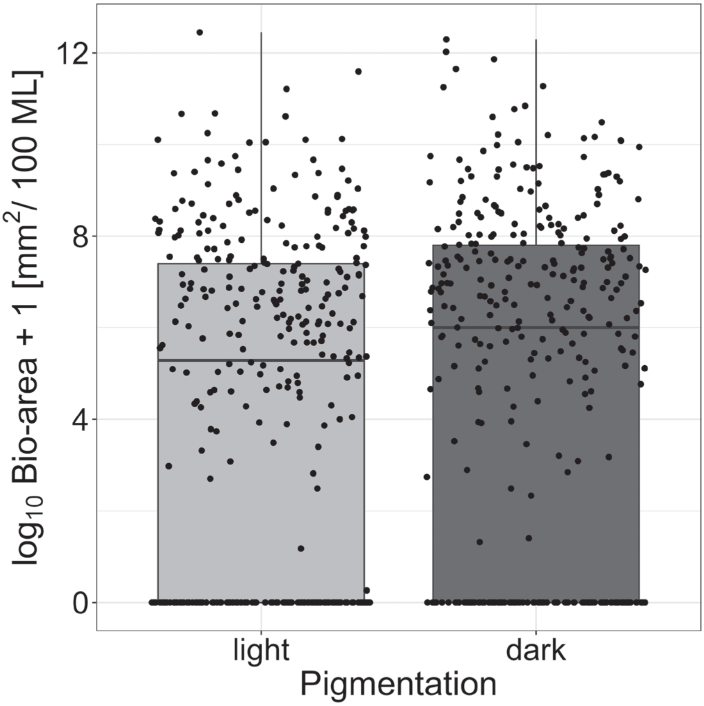

Figure 3. Standardized macroinvertebrate bio-area per site and pigmentation. Bio-area in mm^2 per 100 ML discharged groundwater and at $\log_{10}(y+1)$-scale. The thick horizontal lines show the median, the interior of each box represents the interquartile range (IQR) and the vertical lines represent minima and maxima, respectively (1.5 * IQR).

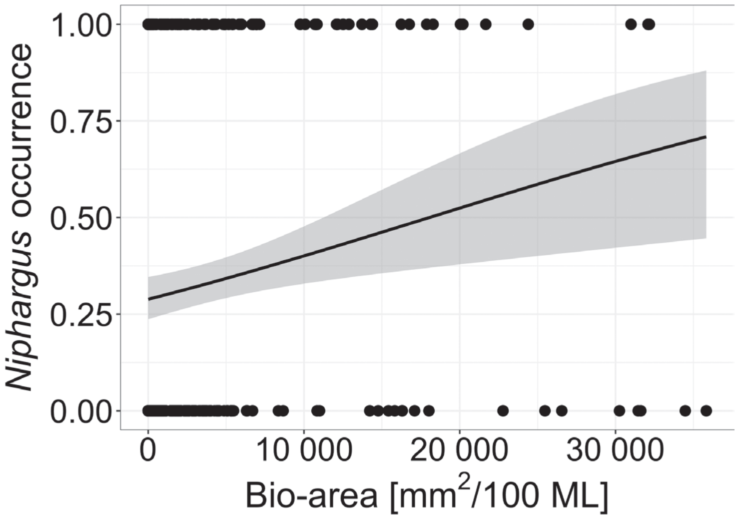

Figure 4. *Niphargus* occurrence in relation to the standardized bio-area of other macroinvertebrates. Predictions of binomial GLM are plotted as solid line with 95% confidence interval (shaded area). The binomial GLM was fitted to 334 sites with bio-area < 40,000 mm^2/100 ML discharged groundwater. Bio-area in mm^2 per 100 ML discharged groundwater.

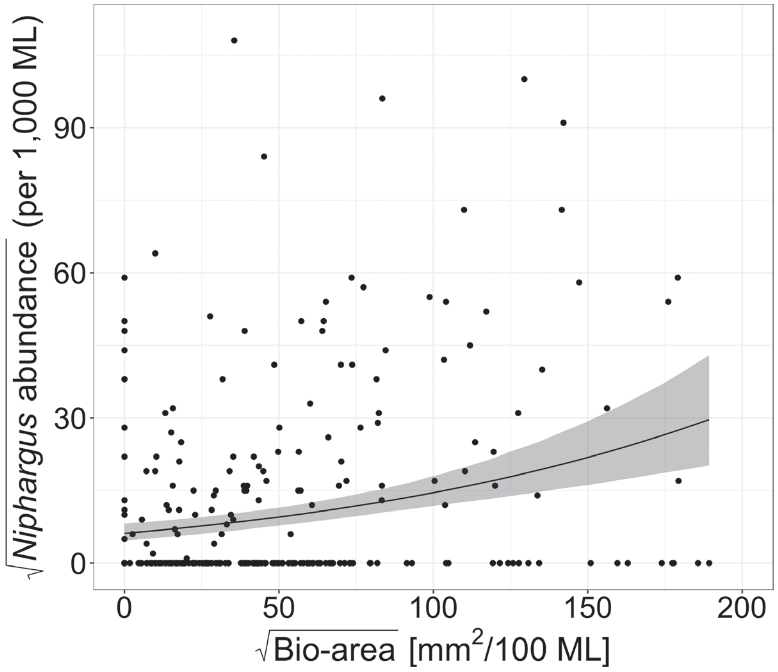

Figure 5. Fit of the ZINB model for *Niphargus* abundance and the standardized bio-area of other macroinvertebrates. Both variables were square-root-transformed. Bio-area in mm^2 per 100 ML discharged groundwater and *Niphargus* abundance per 1,000 ML discharged groundwater. For visualization, only points with values below 200 (x-axis) and 110 (y-axis) are plotted. The 95% confidence intervals are plotted in grey.

Discussion

Here, we provide a first countrywide overview of major groups of macroinvertebrates found in Swiss groundwater systems and address possible associations between groundwater and surface ecosystems through the assessment of these organisms. While groundwater amphipods are relatively well-known for this area (Altermatt et al. 2014, 2019; Fišer et al. 2017, 2018; Alther et al. 2021), the remaining groups have been understudied even at coarse taxonomic scale, hitherto prohibiting a first overview and understanding of their abundance and occurrence. Thus, we herewith contribute basic knowledge needed to successfully protecting and conserving the biodiversity of groundwater ecosystems (Wynne et al. 2021; Borko et al. 2022).

The application of a citizen science approach proved suitable to collect a broad range of macroinvertebrates from shallow groundwater aquifers. Collaborating with local drinking water providers to receive samples from spring boxes enabled us to obtain macroinvertebrates across a large geographic area, and from sampling sites that are otherwise not accessible to the public. In addition, the provided documentation on sampling duration and water discharge allowed highly standardized analyses of the samples. As such, it might be a suitable method to overcome the Racovitzan impediment (Ficetola et al. 2019), by providing large-scale, systematic data on groundwater fauna.

In line with previous studies (e.g., Hahn 2006; Hahn and Fuchs 2009; Johns et al. 2015), we found a high occurrence of macroinvertebrates in Swiss groundwater samples. The two stygobiotic taxa *Niphargus* and groundwater Isopoda (Asellidae, cf. *Proasellus*, unpigmented) had some of the highest absolute abundances. This pattern of high abundances of crustaceans is characteristic for groundwater systems (e.g., Sket 1999; Gibert and Deharveng 2002; Deharveng et al. 2009; Gibert and Culver 2009). Generally, we could associate the obtained macroinvertebrates with a combination of surface and subterranean, as well as terrestrial and aquatic ecosystems (i.e., groundwater, soil, and surface freshwater).

The high abundances and widespread occurrences of macroinvertebrates from surface waters such as Plecoptera, Trichoptera, and Ephemeroptera larvae (EPT taxa), *Gammarus*, as well as groups containing aquatic organisms (e.g., Diptera and Coleoptera larvae) were surprising and could reflect surface water infiltration and the interactions between above- and below-ground ecosystems (Griebler et al. 2010; Stein et al. 2010; Sinreich et al. 2012; Durkota et al. 2019). This result might be explained by the shallow depth from which most pipes drained the groundwater from the aquifers in our study. While some organisms might have been washed accidentally into the groundwater, some of these macroinvertebrate groups likely inhabit subterranean waters occasionally, for example during their larval stages (Stanford and Ward 1993; Malison et al. 2020). Consequently, they influence processes that occur in this ecotone, such as the transport of resources between surface and groundwater systems (Ward et al. 1998; Boulton 2000; Boulton et al. 2008; Barzaghi et al. 2017). Additionally, it has been suggested that some macroinvertebrates from surface waters migrate to the hyporheic zone or even deeper to escape disturbances such as droughts, floods, and pollution (Ward et al. 1998; Boulton 2000; Griebler and Avramov 2015; Durkota et al. 2019).

Local macroinvertebrate diversity and richness at the order level was significantly associated to the aquifer type. Unconsolidated aquifers had higher medians for diversity and richness compared to fissured aquifers. Similar observations have been made for example by Malard et al. (2009), who found that species richness of stygobiotic crustaceans was on average higher in porous aquifers. Previous studies also proposed that local groundwater faunal abundance and diversity is particularly influenced by the hydrological connectivity (e.g., Hahn 2006; Griebler et al. 2010; Foulquier et al. 2011). Therefore, a possible explanation for the observed higher diversity and richness in unconsolidated aquifers could be the fact that unconsolidated aquifers in Switzerland are found mainly along the main rivers of the Swiss Plateau, leading to higher surface water infiltration.

Aquifers are environments that lack photosynthetic primary production, and groundwater fauna relies largely on allochthonous energy sources imported from the surface (Gibert et al. 1994; Humphreys 2006; Foulquier et al. 2011), including organic matter inflows from plant materials, but also the immigration/inflow of invertebrates that can be predated on by groundwater organisms. An essential parameter for studying the transfer of energy between ecosystems is the biomass of macroinvertebrates (e.g., Machuca-Sepúlveda et al. 2022). In this study, we used the pigmentation of the macroinvertebrates as an approximate classification of hypogean (light-pigmented) and epigean fauna (dark-pigmented). This approach allowed us to overcome certain challenges of classifying macroinvertebrates into hypogean and epigean, as it did not depend on detailed taxonomic identification of the organisms. However, we acknowledge that pigmentation fauna can exhibit a range of variations and thus might only give insights into the organism's affinity to hypogean or epigean ecosystems to a certain extent.

Of all the standardized macroinvertebrate bio-area obtained, 40% was classified as light-pigmented, approximating the hypogean fauna (including stygobiotic and terrestrial subterranean organisms).This portion is in accordance with the expected low biomass of groundwater ecosystems based on the limited availability of resources (Hose et al. 2022). Contrastingly, 60% of the standardized macroinvertebrate bio-area obtained was classified as dark-pigmented, which might approximate the amount of epigean, non-stygobiotic fauna found in our samples. Possibly, the high proportion of dark-pigmented macroinvertebrates could indicate a substantial input of allochthonous energy into groundwater ecosystems, including detritus and living organisms. The presence of non-stygobiotic organisms in shallow groundwater aquifers might also raise potential for predator-prey interactions among organisms. Yet, the degree and direction of trophic interactions between stygobiotic and non-stygobiotic organisms are not completely resolved and non-stygobiotic organisms could function as either prey or predators in groundwater ecosystems (Gibert et al. 1994). Since evidence suggests that some epigean species may use hypogean environments to escape unfavorable surface conditions, climate change might lead to altered biotic interactions between epigean and hypogean species (Vaccarelli et al. 2023).

We found a positive correlation between the macroinvertebrate bio-area and the occurrence and abundance of groundwater amphipods (*Niphargus* spp.). This could be due to local small-scale differences causing more organisms being washed out of the aquifers into the pipes at some sites. For example, differences in the porosity of the groundwater systems or differences in the construction of the water drainage infrastructure, such as pipe size or depth from which the pipes drain the aquifers might lead to a higher rate of organisms being washed out at spring boxes (see Korbel et al. 2017 for a similar discussion). However, we did not find any significant difference in the obtained amount of standardized bio-area between aquifer types, which might disprove a possible effect of aquifer porosity. Alternatively, it could be an additional indication of the linkage between ecosystems, where a higher connectivity between the aquifers and the surface (and thus higher energy inputs from surface to groundwater environments) might correlate with a higher abundance of groundwater amphipods (e.g., Venarsky et al. 2018; Venarsky et al. 2023). Our study provides first data on the possibly tight linkage between hypogean and epigean ecosystems. We are aware that the lack of detailed taxonomic identification of the organisms and missing local environmental data limit the scope of further conclusions. Additional studies could reveal further insights into food web dynamics of groundwater ecosystems, for example through stable isotope analysis (Gibert et al. 1994; Saccò et al. 2019). Also, more data on groundwater quality could help to investigate the role of *Niphargus*, *Proasellus*, and other stygobites as bioindicators for groundwater monitoring.

Conclusion

Using citizen science samples collected by local drinking water providers, we identified major taxonomic groups of macroinvertebrates in shallow aquifers of Switzerland. Apart from obligate groundwater taxa, such as groundwater amphipods and isopods, we detected a substantial amount of macroinvertebrates associated with other surface and subsurface ecosystems. We also found a positive correlation between the macroinvertebrate biomass and the occurrence and abundance of groundwater amphipods, indicating a linkage between groundwater and other ecosystems. In particular, shallow aquifers might promote hydrological connectivity between surface water and groundwater. A better understanding of this linkage could help to conserve and manage groundwater ecosystems, especially as anthropogenic effects on surface ecosystems will affect groundwater ecosystems too.

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Supplementary material 1

Bio-area and biomass relationship

Authors: Ana Sofia Schneider, Mara Knüsel, Florian Altermatt

Data type: pdf

Link: https://doi.org/10.3897/subtbiol.46.112569.suppl1

Supplementary material 2

Detailed procedure for macroinvertebrate bio-area measurements

Authors: Ana Sofia Schneider, Mara Knüsel, Florian Altermatt

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Supplementary material 3

Supplementary results

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The biospeleological work of Carl L. Hubbs (1894–1979): an appraisal

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Abstract

Carl Leavitt Hubbs (1894–1979) was a prominent and internationally renowned American ichthyologist whose publications include taxonomic descriptions of several North American blindfishes including the Mexican Cave Characin. His archived personal papers reveal a wide-ranging interest in the biology and evolutionary origins of cave and blindfishes, and his discussions and disputes with colleagues about their taxonomy. He also took opportunities to collect other fauna from American caves during the inter-war decades. Drawing upon his unpublished archive and other relevant sources his biospeleological work is chronicled in detail and discussed in the context of his other work.

Keywords

Carl L. Hubbs, Cavefishes, Biological Species Concept, Biospeleology

> *"For the arriving at the inside of things, the publication of letters is the true method."*
> \- Cardinal Newman

Introduction

Carl Leavitt Hubbs (1894–1979) was one of the twentieth century's most prolific and respected American biologists. He was a prominent and internationally renowned ichthyologist, mainly interested in the systematics and distribution of freshwater fishes of

North and Central America and Pacific marine fishes. His first published paper, which was on Japanese flatfishes, was a classical taxonomic work (Hubbs 1915) and the taxonomy of fishes always remained a central component of his work.

Nevertheless, although known mainly as an ichthyologist his involvement in natural history went much wider. He always retained a broad range of interests: Norris (1974) describes him as a "*modern pioneer naturalist*". His eclectic bibliographic output includes works on marine mammals, ornithology, paleontology, archaeology, zoogeography, climatology, evolution, ecology, and the history of science. He was also involved in conservation and applied fisheries research. From 1915 until he died in 1979 he authored or co-authored more than seven hundred publications and received several prestigious academic awards, including election to the U.S. National Academy of Science in 1952.

Within this copious output of published work, a handful of papers are devoted to blind fishes of hypogean (cave, artesian) freshwater habitats and certain morphologically similar marine species (Hubbs 1926, 1927, 1936, 1938; Hubbs and Innes 1936; Hubbs and Bailey 1947). To biospeleologists it is as the author of these works that his name is most likely to be familiar. Primarily they are descriptions of new taxa (Table 1) but also include conjectural discussions on the evolution and ecology of these animals.

However, his archived personal papers show that his published contributions on blind fishes only partly reflect the full scope of his interest in the topic. He recognized reduction and/or disappearance of eyes in fishes as a widespread phenomenon occurring in some deep-sea species and those inhabiting burrows and silty waters, as well as the subterranean forms. Throughout his career he speculated about the ecology and evolutionary origins of blind fishes, and collected notes and references with the intention of writing a monograph on them. He also became interested in cave fauna other than fishes, whenever he could taking the opportunity to collect invertebrates from caves in the continental USA.

Despite his acknowledged contributions to biospeleology, his overall work in this area has never been highlighted or discussed in depth, either by himself or by others. He was never able to complete the treatise on blind fishes or, for that matter, on evolution and speciation, topics that became a focus throughout much his career. Interestingly he himself said the same of Joseph Grinnell: "*To the end of his days* [he] *kept too busy with special researches to … write books which would bring together and make generally available his highly respected views on the relations between organisms and their environment*" (1943b, p. 466).

Table 1. Cave and other blind fishes described by Hubbs.

Name given by Hubbs and publication date	Current name
Lethops connectens Hubbs, 1926	Still valid
Anoptichthys jordani Hubbs & Innes, 1936	*Astyanax fasciatus* (Cuvier 1819)
Typhliasina pearsei Hubbs, 1938	*Ogilbia pearsei* (Hubbs 1938)
Pluto infernalis Hubbs, 1938	*Ophisternon infernale* (Hubbs 1938)
Satan eurystomus Hubbs & Bailey, 1947	Still valid

Fortunately for historians of science Hubbs donated his papers, including correspondence and notes, to the library of the University of California in San Diego (UCSD) [https://library.ucsd.edu/speccoll/findingaids/smc0005.html]. These include documents from his time at the University of Michigan (1920–1944) and those from his time at the Scripps Institution of Oceanography (1944–1979) at UCSD (Fig. 1).

Drawing upon this extensive unpublished personal archive, his published works, and relevant secondary sources, we document and review his thinking on blind fishes and cave fauna, most of which has never been treated in the literature.

Methods

As primary sources, we examined all the records kept at UCSD. We selected and ordered copies of all that relate directly to biospeleology, totaling 668 documents. We paid particular attention to those dealing with blind fishes described by Hubbs. The selected documents were organized and analyzed according to the sender and the recipient(s), date on which they were written, and the kind of document (letters, cards, handwritten notes, telegrams, newspaper clippings). We also reviewed all Hubbs' original publications on this matter. Museum collection specimen accession data and

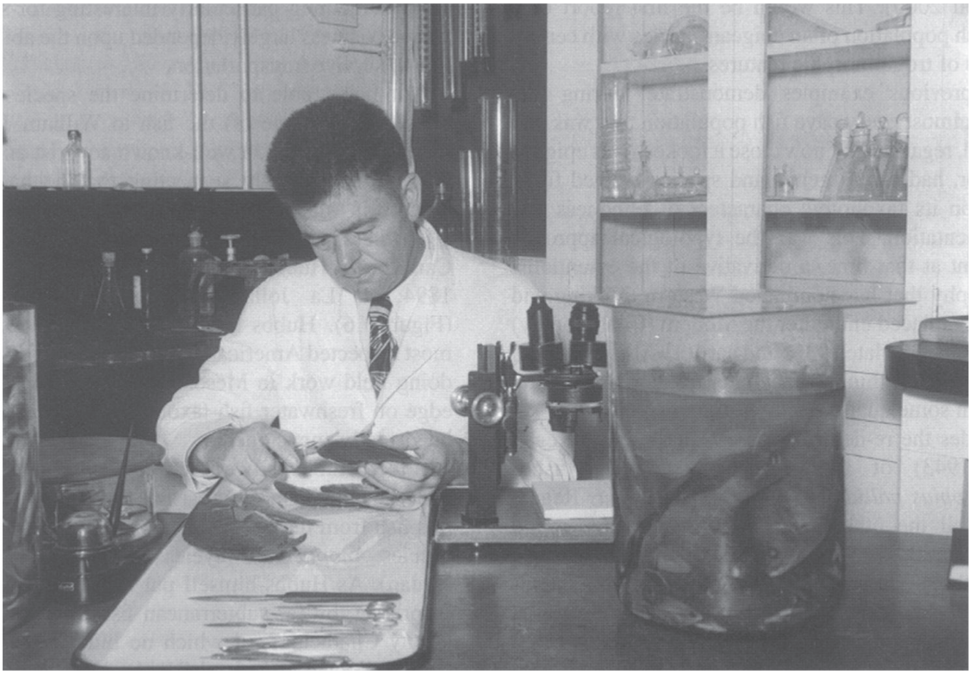

Figure 1. Carl Leavitt Hubbs in his laboratory at the Scripps Institution of Oceanography. This picture was taken in 1945, shortly after he had made his major contributions to hypogean fish research (Photograph courtesy of the Scripps Institution of Oceanography Library).

references in specialist taxonomic works to material collected by Hubbs provided some additional details.

In the following the archived documents are cited by their Group and Box location in the UCSD archives, abbreviated as for example G14B028.

Biographical background

Hubbs was born in Williams, Arizona, on October 19, 1894, moving with his mother to California when still an infant. While living in Los Angeles, an ichthyologist and Junior College teacher, George Bliss Culver (1875–1949), encouraged him to study fishes and advised going to Stanford University, which at the time had become the pre-eminent center of American ichthyology under the leadership of David Starr Jordan (1851–1931). He registered at Stanford in 1913 (Norris 1974, p. 587; Miller and Shor 1997, p. 367).

After completing his B.A. (1916) and M.A. (1917) degrees at Stanford, Hubbs was employed briefly (1917–1919) at Chicago's Field Museum of Natural History (FMNH) as an Assistant Curator. In 1919 he moved (apparently actively recruited) to a curatorial position at the University of Michigan Zoology Museum (UMZM). He was to remain at Michigan for the next twenty-five years, in 1924 taking over the Division of Fishes created in 1920 under the leadership of Walter N. Koelz (1895–1989). He was awarded a doctorate in 1927 on the basis of a paper already in press and overall publication record, and a full professorship in 1940.

Hubbs' next and final career move was to Scripps Institute of Oceanography in October 1944. He died at La Jolla, California, on June 30, 1979.

Fuller accounts of Hubbs' life and career are provided by Norris (1974), Horn (1976), Shor (1979), Shor et al. (1987) and Miller and Shor (1997). Norris (1974) is a particularly sympathetic presentation given on the occasion of Hubbs' eightieth birthday. It is accompanied by a list of Hubbs' doctoral students compiled by his wife Laura (Hubbs 1974) and an extensive selected bibliography (Shor 1974). For a comprehensive indexed bibliography of his published work see Miller (1981).

First encounters with blind fishes

The quarter century that Hubbs worked at Michigan was the period that gave him the most opportunities to study blind fishes. Being inland, the State was an appropriate base for investigating the continental freshwater fish fauna. In addition, it was well situated geographically for travel to the limestone karst regions of Indiana and nearby states. After 1944 when he moved to Scripps Institute, his obligations and focus shifted to the Pacific marine fauna. It is thus perhaps ironic that his first encounter with blind fishes, and first speculations about their evolutionary origins, was not with subterranean forms but with certain marine species endemic to the Pacific coasts of the United States and Mexico.

This first field encounter of a blind fish was with the California Blind Goby (*Typhlogobius californiensis* Steindachner, 1879), a small species ("*circa*" 8 cm) that when adult is a specialized commensal sharing the burrows of a marine crustacean *Neotrypaea biffari* (formerly known as *Callianassa* sp.). It is one of a varied fauna of gobioid fishes found in the littoral and shallow sub-littoral of tidal flats and sandy or muddy bays in California. *Typhlogobius* adults lack eyes and dermal pigmentation: convergent morphological features that it has in common with many cavefishes (Eigenmann 1909 pp. 65–69). The free-swimming juveniles retain rudimentary eyes which is another trait commonly seen in cavefishes (Romero and Green 2005).

It is possible that Hubbs knew of the California Blind Goby from his early years when he was living in coastal California, and even if not, he must have read of it in the work of Carl H. Eigenmann (1863–1927) (Romero 1986b). He presumably became aware of Eigenmann's work on blind vertebrates during his student days at Stanford and was undoubtedly familiar with his publications by the early 1920s. The earliest documented evidence of him searching for a blind fish is a collecting trip to the California coast with his wife Laura in 1922.

In December of that year, the couple collected a single specimen of a previously unknown species that lives in kelp forests but phenotypically is intermediate between *Typhlogobius* and more typical, fully eyed, gobies. The Halfblind Goby (*Lethops connectens* Hubbs, 1926) was described from the type specimen (UMZM Accession no. 63281) and two paratypes (UMZM 63282) that Hubbs collected in May of the following year (Hubbs 1926). The eyes remain functional but become almost rudimentary in the adult, and, while chromatophores remain, no color pattern is evident. Tactile organs are well developed, and scales are absent.

This discovery appears to have been the initial spur of his lifelong interest in the phenomenon of eyelessness in fishes in general including subterranean forms, those inhabiting burrows, silty waters of tropical rivers and estuaries, and deep-sea species. In the year following his description of *Lethops* he published a second paper in which he speculated about the evolutionary origins of this and *Typhlogobius*, drawing a parallel with the North American fish family Amblyopsidae (Hubbs 1927). It was also, in 1924, not long after discovery of *Lethops* that he began investigating cave-associated fishes, collecting amblyopsids in Indiana caves.

The Amblyopsidae

In the mid-1920s the only blind subterranean blind fishes know to occur in North America were amblyopsid cave fishes and a catfish recently described from artesian wells in Texas (*Trogloglanis pattersoni* C. H. Eigenmann, 1919). Amblyopsidae is a small freshwater family (Order Percopsiformes: Trout-perches) distributed in the southern and eastern (unglaciated) United States. The systematics of the family is in flux. Traditional taxonomy relying on gross morphological traits (which result mostly from convergent evolution) has been proven to be unreliable: modern genetic studies have shown that these fishes are much more taxonomically complex than previously believed (Romero 2004).

As currently envisaged, Amblyopsidae is represented by six genera and nine species. Most of the recognized species are exclusively subterranean (stygobites), lacking superficial pigmentation and with eyes reduced or absent. The family exhibits a transitional series from the surface (epigean) Swampfish (*Chologaster cornuta*); the Spring Cavefishes (*Forbesichthys*) which are facultative cavernicoles (stygophiles) inhabiting both springs and caves; and finally obligate cavefishes (*Amblyopsis*, *Speoplatyrhinus*, *Troglichthys*, *Typhlichthys* (Eigenmann 1909; Romero 2004; Adams et al. 2019).

The eyes of amblyopsids range from small (microphthalmic) in the epigean and stygophilic species, to vestigial (remnant eye tissue under the skin) in those living permanently underground (stygobitic). Stygobitic species are also characterized by: (l) depigmentation (they have a pinkish color due to the blood vessels showing through the translucent skin, with only a few, mostly nonfunctional, melanophores); (2) low metabolism; (3) low fecundity; and (4) increased swimming efficiency, tactile receptivity, and longevity.

Cave fieldwork at Michigan 1924

Hubbs' correspondence archive before 1936 is not very informative about the present topic, presumably because his relevant activities, being based in Michigan, rarely involved a need to communicate with workers elsewhere. From 1924 to 1935, his activity has been reconstructed from a few extant field reports, museum accession data, occasional comments in later letters, some surviving correspondence 1931–1935, and published papers.

We do know that in the spring of 1924 he led a small party on a trip to Indiana to collect amblyopsids on behalf of UMZM. They investigated several caves in the limestone district of southern Indiana from the 15th to the 18th of May. Indiana was reasonably accessible by road from Michigan in the 1920s, and several caves in the southern limestone district were already well-known and not difficult to reach and explore. In addition, they already included recorded localities for what was at the time identified as the most common cavefish, *Amblyopsis spelaeus*. Wyandotte Cave is one of the two original localities (the other being Mammoth Cave, Kentucky), and the species had also been recorded in another Indiana cave, Rhoad's Cave (Banta 1907, p. 23; Eigenmann 1909, p. 71). The Indiana population of *Amblyopsis* has been separated recently from *A. spelaea* as *A. hooseri* (Adams et al. 2019).

Handwritten accounts of this collecting trip are preserved in the Hubbs Archives. The most informative is a series of short unsigned reports detailing individual caves visited (G16B028).

There is no full record of the party members. Further details (including UMZM Accession Numbers) of the fishes collected and the caves can be found in Hubbs' notes in archive file G26B29 and Table 2 herein.The party explored the main, higher level of Marengo Cave on the 15th, finding pools but no flowing water. Just before midnight on the 15th, they reconnoitered the lower active stream passage ("Old Town Spring

Table 2. Hypogean fishes collected by Hubbs.

Date	Location	Description	Taxon	Explorers	Specimen	Notes
16.5.1924	Siberts Well Cave, Indiana	Active stream cave in limestone. c. 3ft deep, between pools	*Amblyopsis spelaeus* (Amblyopsidae)	CLH & A.C.Kennedy	UMZM 64997	Near Wyandotte Cave. Also 1 *Eurycea* salamander
16.5.1924	Old Town Spring Cave, Marengo Cave, Indiana	Active stream cave in limestone. Large pools well inside 'long dark cave'	*Cottus bardii carolinae* (Cottidae)	CLH & party	UMZM 54998	Some 'huge'. Living in cave. No eggs or young seen.
16.5.1924	Old Town Spring Cave, Marengo Cave, Indiana	Active stream cave in limestone. Large pools well inside 'long dark cave'	*Semotilus a. atromaculatus* (Cyprinidae)	CLH & party	UMZM 64999	Blind *Cambarus* in pool
16.5.1924	Rhodes Farm cave nr. Croydon , Indiana	Limestone cave. Deep pool, c. 150' from entrance, no current, "thoroughly dark"	*Hyborhynchus notatus* (Cyprinidae)	CLH & E. B. Williamson	CLH, UMZM 64996	Described as a half-grown straggler minnow
17.5.1924	Twin Caves, nr. Mitchell, Indiana	Active stream cave in limestone. Pools in stream, no current	*Amblyopsis spelaeus* (Amblyopsidae)	CLH & party	UMZM 65000	
19.8.1930	River Cave, Campden County, Missouri	Large, deep (<10') clear pools	*Typhlichthys* sp. (Amblyopsidae)	CLH ?	UMZM 156795, UMZM 156796	Associated with pale crayfish *Cambarus* sp. and blind salamamders.
19.8.1930	unnamed cave, Campden County, Missouri	Small limestone cave with fast-flowing stream. Stream, mud bottom	*Cottus williamsoni* (Cottidae)	CLH?	UMZM 102747	
24.9.1931	Jewel Cave, Dickson Co.	Active stream cave in limestone. Pools, mud & lmst bottom, no current, `13.5 C.	*Chologaster agassizii* (Amblyopsidae)	CLH	UMZM 97211	CLH refers to it as *Forbesichthys agassizii*: (CLH to Leslie Hubricht 29th October 1942)

Cave"), entering it for a short distance. Returning early the following morning, they successfully followed it to the end. The same day they visited another active stream cave, "Siberts Well Cave" (the stream outlet of the famous Wyandotte Caverns system) and Rhoad's Cave. The latter consists of a steeply descending passage ending at a deep sump pool. Mitchell Caves and Twin Caves, other parts of the Wyandotte system, were examined on the 17th and the 18th.

It is reasonable to assume that obtaining examples of *Amblyopsis* was the main purpose of the trip. Specimens were secured in Sibert's Well Cave and Twin Caves. He also made observations on and collected specimens of all other fishes seen underground. At least two common epigean species of minnow (Cyprinidae) were present: *Semotilus atromaculatus* in "Old Town Spring Cave" and *Hyborhynchus notatus* in Rhoad's Cave. Both were taken in lightless regions, but Hubbs thought neither to be resident there. Several sculpins (*Cottus bardii carolinae*) including some exceptionally large individuals were living deep inside "Old Town Spring Cave", evidently permanently although no eggs or young were seen. They had been feeding on cavernicolous crayfish. He speculated that the large size reached by these cave-dwelling sculpin (confirmation of a phenomenon previously reported in Indiana cave populations by

Hay [1894]) was due to the protection from predators offered by this habitat. Banta (1907, p. 75) had previously suggested this explanation for the relatively large size attained in Indiana caves by the epigean crayfish *Cambarus bartoni* and the amphipod *Crangonyx gracilis*.

Hubbs may have recalled this example three decades later when interpreting another cyprinid (*Hesperoleucus symmetricus*) that had been collected in Bower Cave, Maripoan County, California and found to be a very old individual (see below).

The notes include observations of the presence of other cave fauna: frogs (*Rana* sp.), salamanders (*Eurycea* sp.), crayfish (*Cambarus* sp.), and various other, unidentified, invertebrates. This is early evidence of Hubbs beginning to take a wider interest in cave fauna in general. By November of 1924 accompanied by a Dr. Jan Metzelar, he was again searching for cave fauna, this time in a small Ohio cave. Evidently, the results were disappointing and all they secured were a few overwintering bats identified as two common species *Pipistrellus s. subflavus* and *Myotis subulatus*.

Hubbs was an inveterate collector amassing huge fish collections at UMZM and later at Scripps (Miller and Shor 1997) and this might well be sufficient to explain why he paid attention to and collected specimens of anything seen, not only the blind cavefishes. However there is significance for his later conjectures in that he approached the animals occurring in caves with the broad viewpoint of a naturalist. He was not constrained in his thinking by a purblind belief commonplace in biospeleology that blind, depigmented animals are the only animals that really matter (or even belong) in subterranean habitats – the only "true" cavernicoles. The focus on these species at the expense of the many other animals that are to be found in subterranean habitats has been termed, admittedly not very elegantly, "troglocentrism" by one of us (Moseley 2007, pp. 1, 11). Rarely made explicit – Weber (2000) is an exception – it nevertheless underlies and pervades the biospeleological literature. This has had some negative implications for the sub-discipline. Romero (2009) noted that this phenomenon may be due at least in part to the fact that it is usual for cave biologists (including the present authors) to have started out as active cavers, hence naturally tending to approach the subject from a 'cave-centered' perspective. Hubbs in contrast should not be considered to be a "caver". He was about 30 years old in 1924 which would have been late in life for a caving enthusiast to begin. Certainly, he focussed his attention on those blind animals that everyone at the time saw as "cave animals" but unusually he also observed and noted that some normally surface fish species appeared to derive benefits from living inside caves.

Discussions of troglocentrism can be found in Romero (2009, Chapter 4) and Moseley (2022, p. 40).

Other cave fieldwork at Michigan: 1925–1944

On occasion over the next two decades, Hubbs visited a dozen or more caves, most of them further afield in the South and West of the United States: i.e. Arkansas, California, Indiana, Missouri, Nevada, New York, Ohio, Oregon, Tennessee, Texas, Virginia, and West Virginia (G29B028 CLH to Don Block, NSS; G16B028; G6B028 CLH to W. Halliday 14.12.1948). He was looking for fish but found only known species. He did add a few new occurrence records (a stygobitic amblyopsid *Typhlichthys* sp. and a sculpin *Cottus williamsoni* [an accidental in this habitat?] in Missouri; and the stygophilic Spring Cavefish *Forbesichthys agassizii* in Tennessee) (Table 2).

Reports and rumors of unknown hypogean fishes were assiduously followed up. The earliest is a February 1934 report from a local resident about Bluegill (*Lepornis macrochirus*) seen in a shallow subterranean stream exposed by a surficial collapse a few miles from Grand Rapids, Michigan. Hubbs took the trouble to obtain the area rainfall records in an effort to explain what had happened. It is unclear exactly what he was looking for, but later correspondence suggests that he was thinking that the fishes had dispersed accidentally during a flood event. The outcome is not recorded in the relevant archive (G28B028). This report had been forwarded to him by a colleague, but by 1937 after some newspaper publicity about the Mexican Cave Characin (see below), he was being contacted by informants directly. File G24028 includes an exchange of correspondence concerning a rumor of a blindfish in a cave near St. Paul, Minnesota. Hubbs suspects it to have been a *Cottus* and comments that, although such reports are worth following up, they usually turn out to be blind alleys (8.2.1937 CLH to Prof. King: G24028).

His notes and letters show that he was no longer collecting exclusively to obtain fish specimens for UMZM. Although this remained the primary purpose he realized quickly that little was known about the biology of North American caves. There is a note to this effect in the files (G16B028). The note is undated but undoubtedly comes from this period. Presumably motivated by this, and probably also by simple curiosity ("*I can hardly resist the temptation to go into* [caves] *on passing*" CLH to A. W. Reese 7.3.1933) he also made notes on and collected other vertebrate and invertebrate fauna that he saw in the course of his underground perambulations. These were not methodical surveys: he just secured those obvious, larger animals he saw in a single visit. Nevertheless, this was pioneering work that has been overlooked in the literature: there was almost no other general cave fauna collecting in the United States during the inter-war years. Biospeleology there was "*effectively dead*" (Romero 2009 p. 41). Some specialist taxonomists were certainly interested in receiving and studying cave material but rarely explored caves themselves. For the most part, their work was purely descriptive, confined to occurrence records and reports of new species: one minor exception was a speculative discussion of the evolution of cave isopoda (Miller and Hoy 1939).

It was very different in Europe, where Emil G. Racovitza (1868–1947), René Gabriel Jeannel (1879–1965) and others were engaged in evolutionary theorizing and extensive international surveys of cave fauna (Romero 2009, pp. 50–51). Perhaps reflecting the pre-War isolationist political climate the Americans worked in isolation. The archives show Hubbs corresponding with prominent ichthyologists in Europe and elsewhere but there is no mention of any cave biologist or indication of awareness of their work.

Hubbs had no invertebrate taxonomy expertise, and he did not publish results himself: "*I have done quite a bit of collecting in caves, but .... have published only on* *fishes.*" (CLH to Edward Danby 22.8.1950: G1B028). His material went off for examination and when appropriate, taxonomic description by specialists. Descriptions and occurrence records are scattered amongst specialist journals and difficult to trace. Several previously unknown subterranean species were obtained including two spiders (*Bathyphantes hubbei* Chamberlin & Wilton, 1943, *Archphantes cavaticus* Chamberlin, 1943); a millipede (*Tidesmus hubbsi* Chamberlin, 1943); a crayfish (*Cambarus hubbsi* Creaser, 1931); an amphipod (*Stygobromus hubbsi* Shoemaker, 1942); and a flatworm (*Kenkia rhynchida* Hyman, 1937 [now *Macrocotyla rhynchida*]).

It was not until the nascent National Speleological Society, founded in 1941, began to inspire biological cave surveys that there was a post-war revival and a more systematic approach in America. File G29B028 shows that Hubbs took an early interest in the fledgling NSS, writing to Don Bloch, then Editor of the Bulletin, on 3.4.1944 requesting information on the society's scope and mentioning having had a longstanding interest in cave fauna. Bloch replied 6.4.1944 enclosing a sample Bulletin and invitation to join, which Hubbs submitted (12.4.1944) with a set of separates of his cavefish publications and an offer to write an article for the Bulletin summarizing his overall cave fauna finds. He received his membership card the following week.

Cavefish taxonomy Michigan 1932–1944

Although by the early 1930s, Hubbs had not yet been able to add significantly to knowledge of the cavefish of the continental USA, he was soon to be gifted an opportunity to study and publish descriptions of exciting new forms that turned up elsewhere in North America: in Mexico's Yucatán Peninsula.

The Yucatán comprises a huge lowland limestone karst region with little topographical relief. Because of the low relief dry (i.e. not flooded) caves are thinly scattered. Most of the freshwater is sub-surface, accessible only in the many flooded sinkholes and shafts known as cenotes that are characteristic of the area. Cenotes typically connect with flooded subterranean passages and conduits. Most Yucatán caves are small but extensive systems with several thousand meters of passages and chambers do exist. Due to the scarcity of epigean waters most of the freshwater fish fauna is found in cenotes or in fully subterranean habitats.

In 1932 UMZM participated in a multidisciplinary expedition to the Yucatán Peninsula led by Professor Arthur Sperry Pearse (1877–1956) of Duke University (Durham, North Carolina). The Carnegie Institution of Washington also participated. This major scientific project generated a series of detailed reports by various experts, with Hubbs responsible for the fishes.

By late 1936 a report on the Yucatán cenotes had been published. Hubbs was unable to report any stygophilic species but *Rhamdia guatemalensis* (Heptapteridae: Four-barbel catfishes) collected from caves by Dr. Edwin Phillip Creaser (1907–1981) (see collection details in G26B29] seemed "*to approach the typical, uncolored, eyeless cave-fishes in their moderate depigmentation and somewhat reduced eyes*" (Hubbs 1936, pp. 166–168, 182–

186). This hint that they are transitional forms is not surprising given his knowledge of the blind gobies and the Amblyopsidae. So, here again (see above), he was clearly aware that it is not necessarily only highly-adapted species that are important in subterranean habitats. This was made explicit in a subsequent paper: "*The not infrequent finding of strays* [sic] *of free-living species in caves shows that caves are very frequently populated with a nucleus from which cave species could theoretically evolve*" (Hubbs 1938, p. 270).

A notorious 'splitter', Hubbs described two cave populations of *R. guatemalensis* as sub-species: *R. g. decolor* (Hubbs 1936, p. 201–203; 1938, pp. 278–280) and *R. g. stygaea* (Hubbs 1936 p. 203–205; 1938, pp. 280–282). Differing from those occurring in the cenotes only in displaying slightly or somewhat reduced eyes and pigmentation, they are unlikely to be anything more than local varieties.

From early June 1936 until August 25th Pearse was back in the Yucatán and now making a specific search for true cavefishes (Hubbs 1938 p. 261; Pearse to CHL 26.8.1936 G27B28). Perceptively he concentrated his effort on caves rather than open-water cenotes. Likely due to the impracticality of exploring physically rigorous deep caves all were small. Of the seven named in Hubbs' later paper the longest (the site where Pearse found the first blind fish) was 260 m (Hubbs 1938). Nevertheless, he was successful.

The strategy of targeting caves was suggested by unconfirmed nineteenth century reports of blind fishes in Yucatán caves, and the 1932 discovery there of stygobiotic crustaceans evidencing the existence of a stygobiotic fauna in the region. It paid off handsomely. On June 8th, probably within days of starting work, Pearse captured a single small blind fish in Balaam Canche Cave, near Chitchén Itzá (Hubbs 1938 p. 291). He must have immediately sent word to Hubbs, who, clearly excited, replied by letter that "*your cryptic news … represents a great rarity*" (CHL to Pearse 18.6.1936 G27B28). The specimen was a juvenile brotulid later designated the paratype of the new species described and initially named as *Typhlias pearsei* Hubbs, 1938 (Table 1).

Pearse also managed to collect a single example of yet another kind of blind cavefish and a second, this time helpfully an adult, specimen of the brotulid. In addition to these were cave-collected examples of a cichlid *Cichlasoma urophthalmus* and more *Rhamdia*. On August 26th after having arrived back home only the previous evening, Pearse sent Hubbs all his material by express mail. In a letter of that same date, he reminded Hubbs that the collecting permit required him to take only three specimens of each species, reserving one for the Mexican national museum: "*Will you please … do what you think is right to fulfill this requirement?*" Hubbs had no intention of giving up the cavefish specimens. His terse reply (16th September) was that "*… we shall proceed to discuss what material we have, with the idea that if we are to lose our collecting privileges thereby, we won't want to continue collecting in Mexico anyway.*" By now, after having had the time to study them he knew that one of the new cavefishes was a brotulid, a normally deep-sea family represented in freshwaters only by two Cuban cavefishes (Romero 2007). The other was an eel of a cosmopolitan family with only one other known cave species (from Africa). "*These blind fishes represent one of the finest ichthyological discoveries in a long time*" (G27B028).

Pearse quickly responded with a short letter requesting a report on these fishes for inclusion in a planned account of the animals of the caves, to which Hubbs readily agreed. Just a week later he asked for guidance where this was to be published so that the UMZM artist could prepare illustrations at the right scale. It took only two days for Pearse to get and forward the information that the Smithsonian had agreed this (G27B028).

Carl Hubbs had been gifted an exclusive opportunity to publish what was, in his own words, "*one of the finest ichthyological discoveries in a long time*"; to which he himself had not so far contributed. He was clearly under some considerable obligation to the man who had not only gone out of his way to find these remarkable fishes, but had done everything possible to facilitate matters. Pearse had promptly sent them; had put his collecting privileges in Mexico in jeopardy; and had answered queries as quickly as humanly possible. However the cavalier attitude that Hubbs had shown towards the Mexican authorities sometimes extended to his academic colleagues.

This was October 1st 1936. The report on the caves of Yucatán did not go to press until 20 months later, in May 1938, and there is no doubt that it was a failure by Hubbs to complete and submit his contribution expeditiously that was the cause of much of the delay. Between October 1936 and November of the following year Pearse, always courteous but increasingly frustrated, sent a series of letters asking Hubbs about progress and pointing to the urgency. At times he was even close to pleading ("*Have a heart man, and finish up*"). He apparently gave up: there are no further letters from him after November 1937. The matter was turned over to the Smithsonian editorial staff and finally, after further delays due to Hubbs asking for last minute changes to text and tables, the report was published (G21B028; G27B028) (Hubbs 1938).

Hubbs was known for having numerous projects active at the same time in various stages of development, and other demands on his time had taken priority (Miller and Shor 1997, p. 375). Only one of these concerns us here. He initially put the Yucatán paper aside because of a totally new development. By a great coincidence yet another undescribed Mexican cavefish had unexpectedly fallen into his lap. The earliest it is mentioned in the archive is in a letter of 16th September 1936, so he had received it around the same time that he also got Pearse's collection or very shortly thereafter (16.9.1936 CLH to Charles Mohr: G29B028).

His response on receiving this new fish could not have been in sharper contrast to the way he was dealing with the Yucatán fishes. He immediately (and urgently this time) started work on a formal description, laying aside the latter. Abandoning his normal tendency to perfectionism in order to rush to publication, he based the description on a single type specimen; designated two live fish that were not even in his possession as paratypes, and illustrated it with the bare minimum necessary in a taxonomic work– a photograph of a live fish in an aquarium. The decision to use this rather than, as would be normal practice, an illustration of the described holotype may have been because the holotype showed an abnormality of the jaw apparently caused by an old injury.

The paper was in print within a few weeks of receipt of the preserved type specimen (Hubbs and Innes 1936). Described on the basis of lacking eyes and pigment, it was named as a new genus and species *Anoptichthys jordani*. This was typical for the period.

That he was ready to postpone work on the important Yucatán fishes, and at the same time risk, perhaps permanently, relationships with loyal colleagues shows the priority he attached to the new species. This episode throws considerable light on his attitude in later years to "his" new blind fish. A particularly telling indication of his somewhat narcissistic claim is a later statement "… *my recent discoveries of five new blind fishes in the caves and artesian waters of North America*" (Hubbs 1940, p. 203). In fact he had not, other than in the loosest sense possible, 'discovered' any of them.

Historical context

The discovery of "*Anoptichthys jordani*" was to prove a milestone in hypogean fish research, and by extension in biospeleology.

Romero (2001, p. 44) distinguished six phases in the history of hypogean fish research: (1) pre-Linnaean 1541–1742, (2) first discoveries and research 1805–1854, (3) American Neo-Lamarckism 1868–1919, (4) dominance of typological thinking 1921–1940, (5) American renaissance 1936–1960, and (6) philosophical conflict 1960–1990. Phase 4 is characterized by incremental discovery and description of new species/populations most of which were assigned generic status solely on the morphological (typological) basis that they lacked eyes and superficial pigmentation (Romero 2001, p. 59). As already touched upon, interest in invertebrate cave fauna in general in North America mirrored this pattern.

Although the discovery of new hypogean fishes continued elsewhere (except in Europe) American-born scientists doing field studies outside the United States and experimental work in American institutions initiated a renewed interest in the subject, mainly because aspects other than taxonomy and morphology began to be investigated. This renaissance period is characterized by more comprehensive studies that included ecology, physiology, and behavior. This was partly because of the discovery of "*A. jordani*" in 1936.

By 1936 only 16 species of blind cavefishes had been described (Romero 2001). None were characids, and except the still unpublished Yucatán forms that Hubbs was working up, only amblyopsids and the catfish *T. pattersoni* were known to occur in North America. Things started to change that year when the new cavefish that Hubbs was to describe was discovered in Mexico (San Luis de Potosí state, central Mexico) in an accessible cave known as La Cueva Chica ("The Little Cave"). The precise circumstances leading to this discovery are not reported in contemporary sources and remain unknown. We know that Señor Salvador Coronado, a young Mexican in charge of the Fish Culture Station at Almoloya del Río near Mexico City, entered the cave and reported the find. The discovery, made on or about 1st November 1936, was not fortuitous. His report, translated from the original Spanish by Hubbs, states "… *to see the little fishes for which we had come*" (Hubbs and Innes 1936 p. 2). Local people from a nearby village were using water from the cave pool where the fish were found (Breder 1942, pp. 8, 10) so presumably they were the original source of the information. What is recorded is that he visited the cave more than once, caught a hundred fish and without delay live-shipped 75 of them to Charles Basil Jordan (1902–1989) proprietor of the Texas Aquaria Fish Company in Dallas, Texas.

Jordan was responsible for bringing to the market several new tropical fish species from Mexico (Hubbs and Innes 1936 p. 1). Coronado's action in quickly providing a commercial firm with so many specimens of a remarkable and unique discovery – three-quarters of the animals he had collected – has the hallmark of a well-established and trusted relationship. This implies that he was a regular collector of Mexican fishes on behalf of Jordan.

The abundance of these cavefish was unprecedented. After all, almost all vertebrate cave populations (except for bats) tend to be small. In addition to their abundance, Coronado found them easy to collect using an aquarium net to scoop the fish out of the water.

Jordan thought those blind and pinkish animals were a fascinating novelty. He was also impressed because all of them arrived in Texas alive and healthy: particularly significant for someone whose business was largely dependent on the ability of live fish to survive transportation (Romero 1986a). Unable to determine the species, Jordan sent some fish to William Thornton Innes (1874–1969), a well-known aquarist and aquarium writer and publisher. Strongly suspecting that he had a new species in his hands, Innes remitted in November 1936 specimens together with Jordan's notes to Hubbs. Unknown to Hubbs, one of his contemporaries, Charles Marcus Breder, Jr. (1897–1983), Assistant Director of the New York Zoological Society, had also received some of the fish, and in a letter, Breder wrote to Hubbs: "*From the letter to Dr. Turner, I note that you were describing a blind brotulid from Yucatán. It occurs to me to tell you that Miss LaMonte* [i.e. Francesca R. La Monte (1895–1982)] *and myself are describing a blind cave Characin from Texas. What do you think of that? We are quite excited. If you have heard about the thing, I would be glad to get any additional gossip you may have on it*" (G26B028: 14.12.1936).

If Hubbs' immediate response upon receiving the letter and specimen from Innes implies that he attached the utmost importance to this new fish, his apprehension on receiving Breder's letter that he might have been pre-empted must leave no doubt. He was quick to respond (G28B028; 16.12.1936). First, he corrected Breder by telling him the fish was from San Luis Potosí, Mexico, not Texas. Breder may have thought that the fish in question was from Texas because Jordan lived there. Hubbs continued to explain that he had received a specimen from Innes, who initially thought of publishing its description, to which Hubbs wanted to keep the priority of the description because: "*I have had very great interest in blind fishes and Middle American fishes.*" He said that the description was already in press and hoped that nobody else had beaten him on that, expressing fears that, in the past, that had happened. Afraid of losing the primacy of describing the species, he had rushed the manuscript with most unusual expediency. The date of publication, 17th December 1936, is a mere six or seven weeks after Coronado 'discovered' the fish. Many of Hubbs' papers show him delaying responding to correspondence, submitting manuscripts, and proofreading them.

Very courteously, Breder replied: "*It's all yours. About an hour after your letter got off, we heard from Jordan, with words that led me suspect (sic) you were doing something; consequently, we stopped then, pending hearing from you. It strikes me that this is a particularly interesting find, and we may try to do something with it here. I would like to know what your future plans are, if any, so we don't cross wires.*" (G26B028; 16.12.1936).

Hubbs followed up by telling Breder the scientific name given to the fish, that the publication date was "*today*" and that the publisher, Ann Arbor Press, was rushing it. There is no question that he wanted to make absolutely sure that he had priority over the description of what he thought was a remarkable new genus and species of cavefish. He continued by saying that he had no plans for further work on the fish beyond systematics and that Innes would soon publish a popularized version of this discovery (G26B028; 16.12.1936). Breder immediately replied: "*Just to keep the record straight: we labored under the impression here that the fish had not been distributed elsewhere, and it was not until after writing you that we got his (Jordan's) somewhat ambiguous note which caused us to stop our description*" (G25B028; 18.12.1936).

Breder sent Hubbs a copy of a letter he received from Innes dated 18.12.1936 in which Innes says: "*Dr. Hubbs has sent me a carbon (copy) of his letter to you of December 16th. There is one point in it which is not quite clear to me, and since I can get a reply from you, I am writing to ask you about it. This is how far has your work advance and is there any question at all about Hubbs having priority. You see, I am planning to publish an article in the forthcoming issue of THE AQUARIUM* (sic), *and in it I have left a blank space for the title and date from Hubbs' paper, which should have been out before this. Without being certain at this point, I would feel I must withhold publication for another month. (…) In any case, I could act more satisfactorily to myself if I knew your wishes and plans. As a matter of fact, I expected the Hubbs' paper to be printed within a few days of the then date, that I wrote Jordan saying that I thought it would be all right to send fishes to you and to Shedd, without danger of confusion in naming. Fowler wanted me (sic) to rush out a description about two weeks ago, but I thought this would be an impertinence on my part, especially as I learned next day that Hubbs was interested. I then sent my photographs to use in his paper, together with all information that I had. He has used my name only by courtesy.*" Henry Weed Fowler (1878–1965) was Curator of Fishes, Philadelphia Academy of Natural Sciences. By urging a rushed description he must have recognized the importance of the new fish, but it is unclear why he thought that Innes, who was a publisher not a taxonomist, might be able to do it.

Innes added a handwritten note at the top of the letter that says: "*Carl, althou (sic) the collector is obligated to keep specimens for the Mex. Govt' (sic), I do not think Breder's source of supply was from these. Jordan, after your paper was well underway to press, asked me if I thought it* [illegible] *to send to Battery Park and Shedd, I said yes Maybe I was rush (sic)*" (G25B028; 18.12.1936).

Although he had acted perfectly ethically, Breder was in damage control mode with a colleague and friend who was jealous of potentially losing priority over the discovery. The confusion had arisen because the businessman Jordan, likely unaware either of the especially great scientific potential of the find or of the fundamental importance of priority in academia, did not inform either one about where he had distributed specimens. But Hubbs and Breder were just some of the ones involved in this confusing race for priority.

Hubbs and Innes described the new species as *Anoptichthys jordani* (Jordan's eyeless fish). As Hubbs put it himself, this was "*most surprising, by far subterranean fish belonging to the family Characidae, of which no blind representative has ever been seen before*" (Hubbs and Innes 1936).

A few days later, on 29.12.1936, C. Basil Jordan sent a telegram to Hubbs to thank him for "*favors accorded in your papers*" in an apparent reference to dedicating the fish's scientific name to him. Hubbs replied in a 2-full-page manuscript letter thanking him for sending four fish that arrived alive. He added some behavioral observations and mentioned that not all individuals look alike. He suggested that some may be the result of hybridization with the eyed relative "sardina" *Astyanax fasciatus* (G25B028; 31.12.1936). These preliminary observations would later become the source of many discussions on the evolution and taxonomic position of this blind cavefish.

The discovery of this new fish attracted the immediate attention of other researchers. For example, Alfred C. Weed (1881–1953), then Curator of Fishes at the FMNH in Chicago, wrote a letter on 5.1.1937 (G25B028) to Innes saying that he was astonished that "*… a fish of this group and closely related to the common* Astyanax fasciatus mexicanus *of southern Texas should get into an underground water system and then become blind. The Cichlids of the Cenotes of northern Guatemala and southern Yucatán which apparently also inhabit underground waters do not seem to show any signs of losing their eyesight.*"

This letter was addressed to Innes and not to Hubbs, who was not only the first author of the paper but also the true professional ichthyologist of the two and someone who had already described fishes from the cenotes. Perhaps Weed was unwilling to communicate with a man who had been dismissed for insubordination from his position as an Assistant Curator at the Field Museum (Norris 1974, p. 588). In any case, Innes provided the letter to Hubbs, and, as might be expected, there is no evidence that Hubbs ever wrote Weed on this issue. Yet, Innes did reply to Weed. In his 7.1.1937 (G25B028) letter he thanks Weed for his interest and gives him some behavioral information about the two captive individuals and why he thinks there is great potential for scientific studies.

Another observation would fuel the idea of the fish becoming an excellent subject for study. In a 10.1.1937 letter to Hubbs, Jordan confirms that he had successfully crossed the cavefish and the surface forms of the fish and that the resulting fish (F_1 generation) show intermediate features in terms of eye development (G25B026; 10.1.1937). After hearing this from Basil Jordan, Hubbs replies to him in a 25.1.1953 letter that the positive cross of the two forms is "*extremely exciting news*" and asks that if the result of that cross survives, he would like to examine the eyes of the preserved specimens (G25B028; 25.1.1953).

The news of the discovery started to attract attention from unexpected quarters. For example from Albert Moore Reese (1872–1965), a professor of zoology from West Virginia University who was working on venomous snakes and antidotes. Reese wrote Hubbs asking if someone was working on the anatomy of the sense organs of the fish (G25B028; 23.1.1937). Hubbs answers by postal card telling him that Breder is interested in doing so (G25B028; 27.1.1937).

By now, the scientific and aquarium communities were impressed about the fish Hubbs and Innes described, but also because so many individuals of several cavefish species in a single locality; the amblyopsids, by that time the best-known cavefish family, were not so abundant. Second, the fact that all 75 individuals had arrived in the U.S. alive and were easily kept in captivity said something about the potential of this species as a research subject (Innes 1937). Third, this cave characin did not grossly display the hyperdeveloped sensory organs quite common among other cave animals. Fourth, it only differs from its likely ancestor, *Astyanax fasciatus*, in lacking eyes and pigmentation. Fifth, initial crossings showed that Mendelian genetics studies of this fish and its presumed ancestor were feasible and promising in revealing some aspects of its evolutionary history.

This cave fish was so intriguing that in both Mexico and the U.S. a great deal of interest arose. So, a group from the Mexican "Escuela Nacional de Ciencias Biológicas" composed among others, by José Álvarez del Villar (1908–1986) and Osorio Tafall (1902–1990), began the exploration of the whole cave system for the area which, as time went by, would yield over 30 cave localities containing this fish.

The other center of interest was in New York City. Only three years after the publication of the description, Myron Gordon (1899–1959), a geneticist on the staff of the New York Zoological Society, visited the cave in which the fish had been discovered. There he collected more individuals which were brought back to New York. Gordon's interest in the fish resided in its lack of pigmentation; after all, fish pigmentation had been his subject of research since the beginning of his scientific career in the late 1920s.

The lack of another structure, eyes, became the interest of another New York-based scientist: Edward Bellamy Gresser (1898–1951). Gresser was a practicing physician and a professor of ophthalmology at New York University. He would use the laboratories of the New York Aquarium beginning in 1936 when first Mexican Cave Characin arrived.

Hubbs was clearly satisfied with the prize of naming and describing a major discovery and did no further direct work on it. He did follow up and exchange views and ideas with others, but it was Charles Breder who took the most active research interest in it (Atz 1968; Romero 1984).

The Aquarium cave expedition to Mexico

In 1939, in collaboration with Gresser, Breder took the initiative of organizing and leading an expedition to Mexico to undertake field studies, obtain enough ecological information for a cave habitat display for the Aquarium, to shoot a documentary to be presented at the 1941 annual meeting of the New York Zoological Society and, most importantly, to bring back enough fish to conduct extensive laboratory research.

In January 1940, he met with other scientists, in which the expedition, known as "The Aquarium Cave Expedition to Mexico," was organized. By March 11th of that year, the group was already in Ciudad Valles, near La Cueva Chica, the fish locality.

In addition to Breder and Gresser, the other members of the expedition were:

- Stanley Crittenden Ball (1885–1956), curator of Zoology at the Peabody Museum of Yale University.
- Marshall Bishop, assistant in Zoology also at Yale and an experienced fish collector.
- Ralph Friedman (1904–1979), an archeologist of the New York Zoological Society, expected to investigate any track of past human activity in the area.
- William Bridges (1901–1984), curator for publications for the Society since 1935.
- Sam Dunton (1912–1976), a professional natural historian and photographer, then working for the Aquarium.

In Mexico, they would be joined by the "discoverer" of the fish (Fig. 2), and by Ramón Aguilar, a local English-speaking native who worked for the Mexican Department of Fisheries.

Besides the fact that most expedition participants suffered from "tropical fevers" (possibly histoplasmosis) after the trip, the expedition was a complete success. The narrative of the expedition has been extensively told in several long articles by Bridges (e.g., 1940, 1954). In addition, the amount of knowledge produced after this and other contemporary field trips to that area has been impressive.

Between 1940 and 1954, Breder, sometimes co-authoring with his wife Priscilla Rasquin (an American Museum of Natural History ichthyologist) or Gresser, published

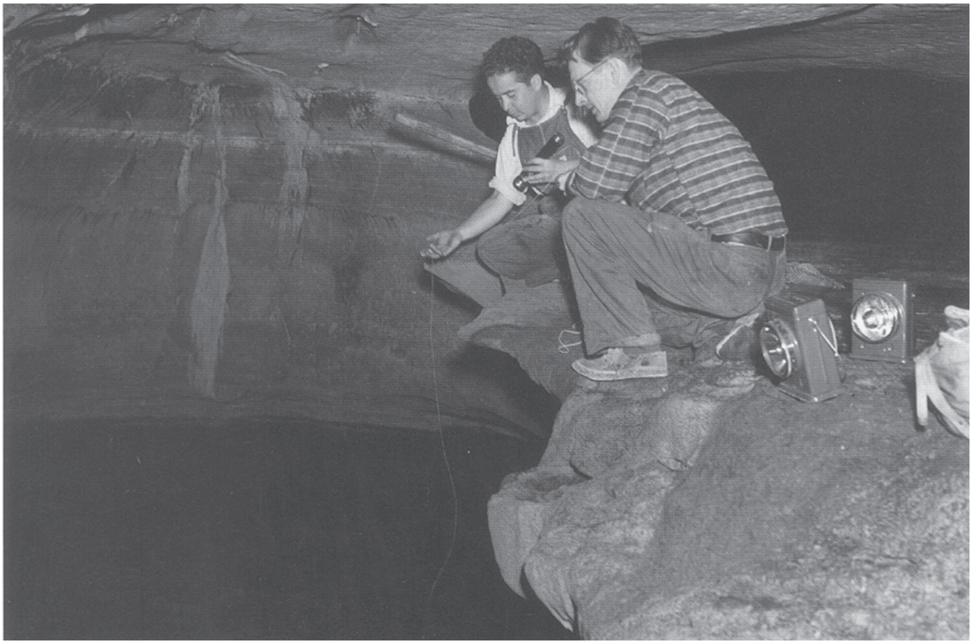

Figure 2. Charles Breder (right) with Salvador Coronado in La Cueva Chica plumbing the depth of Pool 1, the site of the first discovery of the cavefish. The photograph was taken during the 1940 N. Y. Aquarium Cave Expedition to Mexico (New York Zoological Society, courtesy of J.W. Atz).

17 papers (148 pages of dense scientific information) in which this fish was the principal research subject (e.g. Gresser and Breder 1940; Breder and Gresser 1941; Breder and Rasquin 1942). Most of the work concentrated on its behavior, particularly responses to light, chemicals, and social behavior (schooling and karyotypic). He also made the most valuable contributions to our knowledge of this fish's sensory organs (eyes and pineal gland), metabolism, ecology, genetics, and evolution. Based on his observations of the cave populations and because the cave and the surface forms freely interbred, he was the first who strongly suspected that the blind depigmented cave fish was nothing more than a remarkable locally-adapted population ("ecotype") of the surface species *Astyanax fasciatus mexicanus*, long before modern techniques such as electrophoresis and karyotypic analyses were fully developed (Romero 2001, pp. 63–64). By the 1970s genetic analyses had convinced most biologists that this was correct. Hubbs, however, was strongly opposed and remained so until the end of his life (Fig. 3).

Carl Hubbs on evolution and the origin of blind fishes

Throughout his career Carl Hubbs was a consistent supporter and advocate of Neo-Darwinian evolutionary theory, advancing a selectionist and adaptationist point of view. He referred to Darwin as "*the greatest biologist of all time*" (Hubbs 1941a, p. 74). He was unwavering in belief in the centrality of Darwinian natural selection in the process of speciation, and openly critical of those skeptics who doubted or rejected it as the main mechanism driving organic evolution. Although not being one of the "architects" of the Modern Evolutionary Synthesis (MES), his work has been credited with substantially contributing to its development during the first half of the twentieth century (Ilerbaig 2009). As a pioneer of mass-collecting methods and applied biometrics in studying natural variation, he contributed to the introduction of population thinking in evolution. Studies of variation and hybridization in fishes in the 1920s and 1930s contributed to understanding the problem of speciation and he used his many reviews of published works to advance his own views on the processes of speciation and evolution. Despite this, he never accepted the biological species concept which is considered a pillar of the MES.

Born in 1894, Carl Hubbs grew up and came of age in a seemingly unpromising social and intellectual environment for the incubation of a lifelong Neo-Darwinist. Much of the general population believed in a literal interpretation of the "Book of Genesis": evangelical protestant faiths had reached their heyday in the middle of the nineteenth century but remained a powerful presence in the religious landscape of the nation (Frankiel 1988). Within educated and academic circles acceptance of the antiquity of the earth and the fact of biological evolution was the norm by the turn of the century, but for the most part evolution was interpreted within a Neo-Lamarckian framework (Romero 2009, pp. 21 et seq) or as a progressive teleological process: "*God's way of doing things*". Darwinism, in the sense of evolution by natural selection, was viewed with considerable skepticism. As the prominent Congregational pastor, evolutionary theologian and author Lyman J. Abbott (1835–1922) put it: "*All biologists* [now] *accept evolution; practically, all natural scientists accept evolution ...*

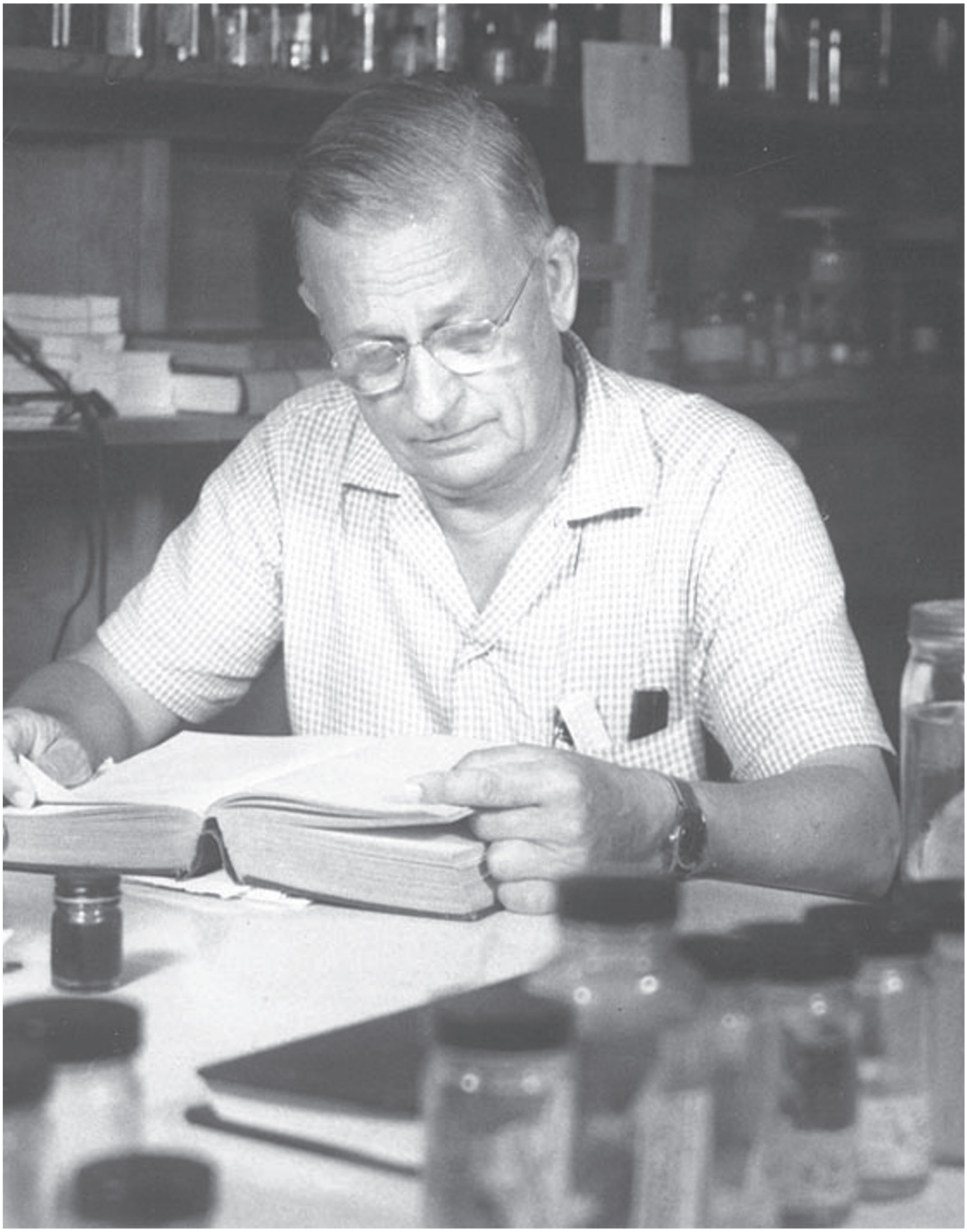

Figure 3. Breder, in later years, working with a fish collection (photograph by M.E. Braden, courtesy of Ms. P. Rasquin-Breder).

Evolution is to-day accepted as the clue in their investigations by all teachers, in all departments, in all colleges and institutions of learning, except possibly in the department of theology" … [but] … "*Darwinism is not evolution, though it is often in popular imagination confounded with evolution. Darwinism stands for the doctrine that the progress of life has* *been due to a struggle for existence in which the fittest have survived and the unfittest have perished*" (Abbott 1897 pp. 95, 177). The first decades of Hubbs' career were during this period famously called "*the eclipse of Darwinism*" (Huxley 1942; Bowler 1988, 1992).

But Hubbs seems to have been unaffected: a story he told in a talk given at Scripps Institution in 1974 reveals that even at a young age he already accepted organic evolution as a fact of life. He recalled attempting when still a schoolboy to produce an illustrated phylogeny of the Mollusca (Shor et al. 1987, p. 219). The explanation of his independence of mind might be found in a free-thinking family who for example had sympathies with unconventional metaphysical religious movements. Objecting to the strictures of the public school system, his mother enrolled him in a private school run by Theosophists. He was there for three years.

Hubbs' early work on fishes focused on intra-specific variation through analysis of correlations between meristic variation, geographical distribution and physical conditions (Hubbs 1918, 1921a, 1921b, 1922, 1924). His doctoral dissertation reviewed this work; first deriving general conclusions and secondly drawing attention to some possible implications in evolution, speciation and phylogeny (Hubbs 1926b). He argued that these local morphological variations are best interpreted as the result of what he called adaptive physiological differences: "*The role of adaptation in evolution is probably more extensive than it has generally been held to be in recent years*" (p.69). However the ideas he expressed about evolutionary implications had little impact. Google Scholar (Accessed 25th May 2023) lists 178 citations of the paper. Most are in works on ontogenetic processes and phenotypic plasticity with very few citations in publications directly dealing with evolution.

He next turned his attention to a study that was to contribute his most significant and novel finding. Before going on to this, it is worth noting that the 1926 paper has a section dealing with the problem of "*degenerative evolution*" (pp. 70–72, 77). It does not mention blind fishes but may have a bearing on his later thinking about their evolution.

By 1930 Hubbs had begun a study of hybridization in fishes, work in which his wife Laura (1893–1988), a mathematician by training, fully collaborated. Their first co-authored paper was on their experimental demonstration of hybridization in sunfish, evidence for the existence of viable natural hybrids in fishes, a possibility previously disputed (Hubbs and Hubbs 1932). This was followed by demonstrations of the phenomenon in other species and even the existence in some cases of inter-generic hybrids.

The significance of hybridization in fish speciation and evolution was hardly recognized then. When "The New Systematics"(edited by Julian Huxley) appeared in 1940, Hubbs was mentioned only once, briefly. Recognition had to wait. A later comprehensive review of natural hybridization between fish species is by far his most cited publication (Hubbs 1955). Google Scholar (accessed 25th May 2023) reports 1151 citations. A search through these confirms that the significance of hybridization as an evolutionary mechanism in fishes is well recognized now.

Thus by 1940 Hubbs had had a major role in introducing population thinking into the study of organisms in nature, had begun to show how the results of such studies help reveal the mechanisms driving speciation and evolution, and in a collaborative effort with his wife, had discovered what has proven to be a significant factor, natural hybridization.

In 1941 "The American Naturalist"added a new "Reviews and Comments"section. As its first editor Hubbs focused on reviews of publications dealing with evolution and on other items of interest to those working in that field: "*Emphasis is given to books and major articles which fall within the special scope of* THE AMERICAN NATURALIST *in that they deal with the factors of organic evolution.*" Until the journal "Evolution"appeared in 1947, "The American Naturalist" was a valuable forum for American biologists interested in the subject, and Hubbs took full advantage of the opportunity this offered him as an influential platform on which to promote his own views. Throughout the years of his editorship (1941–1947) he expressed those views in a series of reviews of published works. These reviews may well constitute his main contribution to the MES. In the absence of any monographic work, they offer picture of his thinking through the 1940s, the crucial period during which the MES was firming up and becoming established. It is generally considered to be complete by the end of the decade.

The review of Julian Huxley's "Evolution: the Modern Synthesis "is introduced with "*It would probably be no exaggeration to call this the outstanding evolutionary treatise of the decade, perhaps of the century. The approach is thoroughly scientific; the command of basic information amazing; the synthesis of disciplines masterly*" (Hubbs 1943a p. 364). The extent of agreement is emphasized by the fact that most of the review consists of text quoted *verbatim* from the book with explanatory introductory comments added. Selection and adaptation are central: "*Evolution is a joint product of mutation, recombination and selection*" "*Adaptation is omnipresent*". Lamarckian explanations are rejected: "*The Lamarckian interpretation is neither necessary nor tenable*" (Huxley [1942] quoted in Hubbs 1943a, pp. 365–366).

Hubbs is dismissive of those biologists who accept the truth of evolution yet contest Darwinian natural selection as the sole or primary mechanism. He was particularly critical of the saltationist theory espoused by the German-American geneticist Dr. Richard Goldschmidt (1878–1958) which he attacked in several reviews e.g. Hubbs (1941b, 1945). In reviewing Goldschmidt's 1940 book entitled The Material Basis of Evolution he is especially critical of the distinction made between microevolution and macroevolution (Hubbs 1941b).

The somewhat eccentric "Age and Area" theory proposed by botanist J. C. Willis was another target. Like Goldschmidt, Willis favoured saltational evolution. He questioned the adequacy of natural selection of small chance variations as the main mechanism, turning to "*… a compelling internal force 'differentiation (orthogenesis)' and regards it as 'a kind of compromise' between special creation and natural selection*" (Hubbs 1942, p. 96).

Turning now to his specific speculations on the evolutionary origins of blind fishes, we have seen how the roots of his keen interest can be found in the 1922 discovery of the Halfblind Goby when he was 26 years old. Eigenmann had already drawn attention to the similarities of behavior shown by the Blind Goby and other gobies occupying much the same habitat, and also its morphological similarity to cave fishes

(Eigenmann 1909, pp. 65–69). Discovery of *Lethops* enabled Hubbs to postulate an evolutionary pathway from a fully-eyed ancestor "pre-adapted" because of a comparable lifestyle. He was to adopt an analogous model to explain the origin of blind cave fishes. The concept of pre-adaptation can be challenged (Romero 2009 pp. 141 et seq.) but it does imply active colonization of subterranean habitats, which was by no means universally accepted in the 1920s.

The potential for active colonization by preadapted species was also implied in the case of two epigean fishes – a sculpin *Cottus b. bardii* and a minnow *Rhinichthys cataractus* – captured in Sinks Cave, Randolf County, West Virginia. These had been sent by Prof. A W Reese (West Virginia University) for identification. In his response (7.3.1933: G29B028) Hubbs commented that "*both species live largely in swift water under stones, so are logical candidates for cave inhabitants, and good material for cave speciation*".

The relative or total absence of predation afforded by caves is one factor probably involved in the process of active colonization. Hubbs proposed it to explain the large size reached by some individuals in a permanent or semi-permanent population of sculpin in an Indiana cave (see above). That was in 1924. There was another example which came to light much later as a result of the post-War revival of interest in cave fauna within the American caving community.

Hubbs had always followed up rumors and reports of possible new blind subterranean fishes (see above) and became very interested in such reports west of the Rockies. He was therefore immediately intrigued when in December 1953 Raymond deSassure of the Nevada-based Western Speleological Institute sent him a single, rather poorly preserved, specimen of a minnow collected by Jon Lindberg (the son of the famous aviator) in the "Daylight Zone" of Bower Cave, Mariopa County, California (1.9.1953: G30B29). The individual had lost all its pharyngeal teeth – a key diagnostic character in these fishes – but based on other features and geographical locality Hubbs tentatively assigned it to a common local species *Hesperoleucus symmetricus*. He was initially excited by the possibility that it represented a cave-adapted endemic subspecies: "*In that event, this would be the first differentiated cave fish to be discovered in the Pacific drainage*". This optimism was based on the rather thin evidence of its cave habitat, apparently isolated from surface waters, and the observation that the anal and dorsal fins each had one fin ray less than normal. Naturally, he asked if additional specimens might be collected for confirmation (CLH to deSassure 8.12.1953: G30B29).

Copies of his letter went to the collector, Lindberg, and, testifying to the potential importance of such a discovery, also to his close colleagues Wilbur Irving Follett (1901–1992), Curator of Ichthyology, California Academy of Science; Garth Ivor Murphy (1922–2001), a fisheries oceanographer at Scripps; Robert Rush Miller (1916–2003), a University of Michigan ichthyologist; and Phil Cummings Orr (1903–1991), Curator of Paleontology and Anthropology, Santa Barbara Museum of Natural History. There was also a direct request to Jon Lindberg for, if possible, the collection of further material (14.12.1953: G30B29).

In response, deSassure confirmed from personal knowledge that the cave lake was almost certainly completely isolated hydrologically, and that another, better specimen of the minnow had already been collected some months earlier and sent to Dr. Follett. It was actually Follett who had originally identified Bower Cave as a possible site for cave fishes and three years earlier had examined it, initially with negative results (16.11.1950: Follett to Danby, G1B028). The site was attractive for exploration and Lindberg made an exploratory dive there using SCUBA equipment, finding an extensive underwater cave and capturing a single fish specimen (8.4.1953: Orr to Hubbs, G1B028). Follett's identification of this specimen as *H. symmetricus* confirmed Hubbs' preliminary opinion based on the new example collected during a second dive (17.12.1953: G30B29).

In the meantime Hubbs had had the opportunity to meet with Follett and together they had compared the cave specimen to examples of the local subspecies collected not far from the cave. They concurred that it did not differ in any substantive way. He conjectured that his specimen was merely an old individual that had lost its pharyngeal teeth with age (CLH to deSassure 21.12.1953: G30B29).

Despite this, Hubbs remained interested (further evidence that he saw all cave-collected fish including normally epigean forms as worth investigation), and asked Follett if deSassure could collect more specimens (CLH to Follett 21.12.1953: G30B29). DeSassure had already told the latter that only a few more might safely be taken because the isolated colony appeared to be small (deSassure to Follett 20.12.1953: G30B29). Interestingly it was *deSassure* who, in a reply to Hubbs' suggestion that his specimen was an unusually old individual, pointed out that, as the topography of the cave offered protection from predators it could have survived longer than usual for the species (9.1.1954: G30B29). Hubbs claimed to have already had the same idea (undated [1954]: G30B29).

There was an addendum to this exchange of letters when Jon Lindberg himself contacted Hubbs with further information about the collection site, and recommending against taking further specimens because very few fish had been seen (13.1.1954: G30B29). Hubbs replied with thanks and that must have been the end of the episode (27.1.1954: G30B29).

To return to the 1920s, Hubbs had speculated about the origins of the blind marine gobies but had yet to say anything specific on the evolution of cave fishes other than linking the unusually large size of cave-dwelling sculpin in Indiana caves with an absence of predators – an observation that has some relevance to the early stages of cave colonization. There is mention of so-called "degenerative evolution" in a speculative discussion of various aspects of fish evolution in relation to differential growth rates, but without direct reference to cave fishes. Presumably, no relevant data was available at the time (Hubbs 1926b). For the next decade he maintained an interest in cave fishes, collecting them whenever he had an opportunity and almost certainly in the unfulfilled hope of discovering new species. But it was not until the mid-1930s that material coming into his hands from others enabled him to begin to speculate seriously about their speciation and evolution.

The two fish species collected in Sink's Cave had been consistent with the hypothesis that blind cave fishes are descended from ancestors preadapted by lifestyle, and there was nothing about the catfishes taken in caves during the 1932 Yucatán expedition that might have challenged this interpretation. Populations of *Rhamdia guatemalensis*, the common "bagres" catfish of the region, were present in cenotes and caves. Specimens from the open cenotes were unusually black; in contrast those collected in caves were partially depigmented with reduced but functional eyes. Based on relative body measurements and gill-raker counts, Hubbs – a notorious "splitter"- described the cave forms as new subspecies *Rhamdia g. decolor* and *R. g. stygia* (1936, pp. 201, 203).

The new Yucatán stygobites that Pearse collected in 1936 were already being worked up for publication when Hubbs received the specimen of the blind fish he was to name *Anoptichthys jordani*. This was a total surprise: a blind cave form living in proximity to an obvious immediate ancestral species which is not in any perceptible way preadapted for subterranean life. "*The discovery of this blind characin was most unexpected, for* Astyanax, *a free-swimming, midwater fish, does not possess the crevice seeking habits nor the well-developed sensory organs that are ordinarily characteristic of the ancestors of blind, subterranean fishes*" (Hubbs and Innes 1936, p. 3). Nothing like this had been found before. It was so remarkable that, as we have seen, other priority work was laid aside so that a taxonomic description could be rushed through to publication (Hubbs and Innes 1936).

The long-delayed paper on the new Yucatán blind cave fishes is the only inclusive treatment available covering Hubbs' views on the topic of blind fish evolution and speciation. In it he casts his net widely, listing all then-known blind forms (excluding deep-sea species). He describes the two new species and amends previous descriptive accounts of other relevant forms while also speculating on their evolutionary origins and those of blind fishes in general (Hubbs 1938).

"Pre-adaptation" had been a common thread since discovery of *Lethops*. Now in this paper he assembles and summarizes all the evidence which he has to support it as the fundamental concept underlying and explaining the evolutionary history of all blind fishes. All, that is, until now: "*Anoptichthys*" was an anomaly and hence a challenge. But it did not trigger any deep questioning or reappraisal of the concept. It was treated as an exception to the general rule resulting from a special situation. Nevertheless an admission that this "*circumstance indicates that almost any fresh-water fish may have the capacity to become a blind, unpigmented cave form, provided other conditions are favorable for this speciation*" suggests that Hubbs did have an inkling of a much broader issue (Hubbs 1938, p. 271).

The proposed model excludes more diverse colonization routes. It is proposed that caves and other dark habitats are actively colonized by species already moderately preadapted to life in darkness by their cryptozoic or nocturnal habits, typically already with some associated reduction of the eyes, and with tactile and other non-visual sense organs relatively well-developed. By "*moderately*" Hubbs means to exclude the idea sometimes proposed that "*fully*" preadapted blind species colonized caves. Emphasis is placed in this paper on evidence that sense organs such as the barbels of catfishes are necessary preadaptations. This was a new factor that Hubbs had not referred to in previous publications. Reproductive methods were also added and briefly addressed as possible useful or necessary preadaptations.

Only these moderately preadapted species are able to enter and establish viable permanent cave populations. It is worth reiterating here that despite the deficiencies of the concept of preadaptation, it does at least constitute recognition that the colonization of caves is an active process. It tells against the idea that cave animals arise from accidental strays that became trapped or other such passive mechanisms. That Hubbs understood this point is demonstrated by his remark that "*The not infrequent finding of strays of free-living species in caves shows that caves are very frequently populated with a nucleus from which cave species could theoretically evolve. There is little ground for supposing, however, that mere accidental strays have become modified into cave types, for such strays would not likely have been common enough to have formed a breeding stock, or would not have found conditions suitable for reproduction*" (Hubbs 1938, pp. 270–271).

Speciation, a process Hubbs recognized as distinct from colonization, takes place "*within*" the subterranean environment. It is stated to be characterized by reduction and eventual loss of eyes, loss of dermal pigmentation, and enhancement of non-visual sensory organs. However, as will be documented below, it is clear that in practice it is the loss of eyes alone that is the factor that is used to differentiate a new species.

Turning to the mechanisms driving speciation, Hubbs overlooked the obvious role of natural selection in adaptive enhancement of sensory structures, and focused only on what he called "*degenerative evolution*". Rejecting the Lamarckian implications of "*use and disuse*" he considers the proposal that reduction of eyes and pigment confer a competitive advantage by conserving energy, but observes that can hardly be the case with endoparasites, which also lack eyes and pigment. The simplest explanation, he contends, is the survival of what he calls "*mutations of loss*" in the absence of strict natural selection (Hubbs 1938, pp. 270 et seq.). Passive genetic drift as an explanation of troglomorphic atrophy in cave animals had already been proposed by the late nineteenth century (Weismann 1885, 1889). Hubbs evidently was unfamiliar with the European biospeleological literature.

The species question

At the beginning Hubbs took it for granted that here was an entirely new form worthy of the status of a new genus. It was obviously very different in appearance from the related but well-pigmented open-water fish with fully-functional eyes. All the fish collected by Coronado were eyeless and almost completely depigmented, and Hubbs himself had virtually equated the process of speciation in cave fishes with reduction and loss of these two traits. Further, the La Cueva Chica population appeared to be ecologically isolated, potentially an important factor enabling speciation.

The genus "*Anoptichthys*" was described as differing from *Astyanax* only in characters associated with subterranean life, specifically eyelessness and depigmentation. The latter was downplayed: color being treated only as a species-specific character (Hubbs and Innes 1936, pp. 3–7). The genus was, therefore, based on a single, though apparently stable, morphological character i.e., lack of eyes.

Establishing a new genus solely on the basis of these traits was in no way unusual. It was common practice at the time. For example, a number of stygobiotic isopods had been described as species of *Caecidota* on this basis, even though there was little doubt that they were independent lineages derived from multiple ancestral species of *Asellus* (Miller and Hoy 1939).

This of course reflects the prevailing typological species concept, which was not to be widely questioned until the early 1940s with adoption of the biological species concept by Mayr. In the case of blind cave animals, however, there is an additional factor that is usually overlooked: "[eyelessness and depigmentation] *are visually striking to us but the undue emphasis put on them is profoundly anthropocentric and has become ingrained. Witness use in the literature of scientifically meaningless adjectives such as "bizarre" to describe them. Absence of vision and of superficial pigmentation are hardly the most important features enabling an organism to survive in the lightless subterranean. Indeed, it was the fact that they seemed to him not to be in any way essential or even advantageous that so famously puzzled Darwin who, unable to see a "Darwinian" explanation resorted to the vague concept of disuse* (Darwin 1859, p. 137: and all subsequent editions)" (Moseley 2022, p. 40).

However, for some time Charles Breder had harbored doubts about the taxonomy of these fishes. The initial straightforward picture, that "*Anoptichthys jordani*" represented a local subterranean population which had evolved in isolation into a new blind species, had become unsustainable. It had been shown that in aquaria it was able to interbreed with the surface fish producing fertile offspring, and the Aquarium Expedition of 1940 found individuals in the original locality showing all stages of eye development. Then a second population of blind fishes had been discovered by the Mexican group in Los Sabinos Cave, which is approximately twenty-five kilometers north of La Cueva Chica. Fish collected here differed consistently though in relatively minor ways from "*jordani*": these differences are listed in a letter dated 14.10.1942 from Breder to Hubbs (G13B028). Hubbs' interpretation was in full conformity with the model expressed in his description of the Cueva Chica cave fish. He viewed the new fish as another isolated species, proposing to describe it as *Anoptichthys profundorum*. Breder had reservations. When invited to co-author the description he declined, citing his doubts in the 14.10.1942 letter. The relevant part of this letter is worth presenting at length: "*My feelings in regard to the taxonomic status of these things is* [sic] *still in a state of flux, confusion or what have you. The more I find out about them in the lab or otherwise the more flighty my notions become. It is this, of course, that has led to our metaphysical 'arguments'. You seem to have a definite view on the handling of such material … frankly I do not know yet whether I want to call this new form a species, subspecies or let it ride as a genetic phenotype … In the meantime I would rather not commit myself on paper. You may have noted that our various papers have all carefully skirted around the subject which manner of treatment stems from the same mental perturbation.*"

Nevertheless, he goes on to say that because "*profundorum*" differs in more characters from *A. jordani* than the latter does from *Asyanax*, designation as a full species might indeed be justified. He was much less conflicted about the relationship of *A. jordani* to the surface fish having already written a paper stating that the Cueva Chica "*cave characins show complete intergradations with the river characins … and surely represent a single population*" (Breder 1942, p. 14). The following year, he reported that while the first (1936 and 1939) collections of the Cueva Chica fish consisted of only blind individuals, eyed forms had appeared by 1940, and the ratio of eyed to blind had increased by 1942 (Breder 1943b; Romero 1983). This evidence "*would seem to indicate that the blind fish* ***which have been called*** Anoptichthys jordani *by Hubbs and Innes were once separated genetically from the river population of* Astyanax mexicanus … *they have evidently been rejoined by the river population … when they were still able to interbreed freely …* [thus] … *the question of whether the intermediates should be looked upon as hybrids or merely genetic variations of the same stock would seem* [for now] *to be merely academic*" (Breder 1943a, p. 28). Conceivably intermingling "*has prevented the La Cueva Chica stock from* ***evolving into a form that could no longer interbreed with the river fish***" Breder (1943a, p. 30) (Current authors' bold).

In his reply to Breder's 14.10.1942 letter Hubbs does not respond or refer to the latter's concerns, asking instead that Breder reconsider co-authorship (17.10.1942: G13B028).

Sadoglu (1956) and Poulson (1964) supported interpretations comparable with Breder. Then Avise and Selander demonstrated that an analysis of genetic variation strongly supported the view that the subterranean Cueva Chica and surface populations are conspecific (1972, pp. 3, 16). Most biologists now accept this interpretation.

It was not, however, accepted by Carl Hubbs. Despite the increasing strength of the evidence, he remained entrenched in his original belief that they were distinct genera and species, allowing only that others might combine the genera, although he still preferred not to.

The Hubbs Archive is silent on the question of the conspecificy of *Anoptichthys jordani* and *Astyanax* until 1965 when an amateur ichthyologist called Michael Oliver wrote asking very sensible questions about it (29.10.1965: G16B29). Hubbs replied at length, clearly stating his position. Basing his argument on the observation that (so far as was known at the time) intermediates only occurred in the mouth of one cave (i.e. Cueva Chica) they were natural hybrids between the full cavernicolous species and *Astyanax*, which had later reinvaded the cave. It should be remembered in this connection that Hubbs had worked extensively on natural interspecific (and intergeneric) hybridization in fishes and would have found this easy to believe. With regard to whether generic separation was justified, he felt that because genus is an artificial concept, this was a matter of opinion, but he still himself favoured separation (16.11.1965: G16B29).

Shortly after receiving a copy of the Avise & Selander paper, Hubbs sent a very critical letter to John Avise stating that lumping the two species was a "*travesty on biology and commonsense*" and anyone who would do this "*needs a new pair of glasses*." The cave and surface fish are different "*kinds*" (his word) of animal, differing in structure, behavior, habitat and all. Interbreeding is not the only criterion. (20.11.1972:G11B29). Avise replied with a courteous letter pointing out that the results of biochemical investigations must be reported objectively without personal opinions or data based on such factors as morphology, ecology, or physiology (5.12.1972: G22B29). Although perhaps being too diplomatic to say so, Avise must have realized that Hubbs did not understand the work, because his criticisms did not actually address the methodology or results. There was, for instance, no claim in the paper that interbreeding was a criterion for the claim of conspecificity.

It was also rather patronizingly suggested that Avise had been "*a little brainwashed by Mayr*". Evidently, even at this late date when it was becoming broadly accepted within biology, Hubbs rejected the biological species concept. His reasoning constitutes most of his review of Mayr's "Systematics and the Origin of Species"(Hubbs 1943c pp. 175 et seq.).

His position on the nature and taxonomy of the cave characins remained the same. He remained adamant that the surface fish, the Cueva Chica fish ("*Anoptichthys jordani*"), the Sabinos fish ("*Anoptichthys hubbsi*"), and a third population that had been discovered in another cave were distinct taxonomic entities (by which, of course, he implied species). In a 1973 letter to Dr. Jacques Gery, the world's top specialist in characids at the time) he makes this clear: "*I still think that the three types of* 'Anoptichthys' *are distinct entities, even though one of them hybridizes occasionally with* Astyanax fasciatus mexicanus *in one cave mouth!*" (10.9.1973: G10B29).

A letter sent to Basil Jordan, the man who had been so involved at the beginning, is the last mention of this topic in the archive. It makes a nice bookend to the story. Jordan had heard of the name change and wondered about it (21.8.1974: G11B028). Hubbs replied: "*I think that calling* Anoptichthys jordani Astyanax mexicanus *is a downright travesty, as they are certainly not the same kind of beast even if they do interbreed in aquariums, and in the mouth of the cave where the species was first found. There has been a tendency to put blind fishes and their ancestors in the same genus, and that is just a matter of opinion. I would prefer to see* Anoptichthys *retained.*" (29.8.1974: G11B028).

Conclusions

From the 1920s onwards, throughout the period referred to as 'the eclipse of Darwinism' and long before biospeleologists in America began to do so, Carl Hubbs was thinking about blind fauna within a Neo-Darwinian evolutionary conceptual framework. Although he had hoped to consolidate and publish his ideas, he never found the time and had little discernible impact on development of evolutionary biospeleology. In any case he worked in isolation, unfamiliar with the current trends and developments in cave biology taking place in Europe, and the general Neo-Lamarckian and teleological intellectual climate in both there and in the USA would likely not have been receptive at the time.

In a broad 'history of biology' context the case of Carl Hubbs and the description of new species of cavefishes represents an interesting case of conflict between personal prejudices and the emerging scientific consensus within the Neo-Darwinian movement. He was a lifelong advocate for Neo-Darwinism, for example, using his reviews in The American Naturalist in support while criticizing robustly those authors expressing alternative theories of evolution. Recognizing the need to adopt population-based approaches he routinely used mass-collecting methods in fish taxonomy, and was a pioneer in demonstrating natural inter-specific hybridization in fishes. Although not being one of the recognized "architects" of the MES, his work has been credited with contributing to it. Puzzlingly, despite all this, he never accepted one of its most fundamental pillars, the population-based biological species concept.

His difficulty is well-exemplified by the case of the Mexican Cave Characin, a blind fish that was described with a colleague in 1936 as a new genus and species. When new findings began to show that it was merely an ecotype of a common, fully-eyed surface species, he was vehemently opposed, and remained so until the end of his life four decades later. Perhaps to some extent he took it as a personal affront, but it is illustrative of his deeper issue with the definition of a species.

There is no question that the description of this remarkable fish was, for Hubbs, a particularly notable achievement in his career as a classical taxonomist. It is for this reason understandable that he would be disturbed and resist seeing it 'downgraded' to little more than a local variety of a common widely-distributed species. This does not, however, explain his fundamental resistance to the biological species concept, of which this is only one, though very clear, example. In this regard, his use of the word "kind" to express his species viewpoint in the 1972 letter to John Avise is telling. It echoes pre-Darwinian fixism, showing that he still adhered to the views of many of his contemporaries that biological species represent separate distinct 'kinds' – analogous to periodic table chemical elements – and which led to the trenchant criticism of taxonomists as "glorified stamp collectors" as the physicist Lord Kelvin put it (quoted in Gould 2000).

Deeply aware as he was of variation within populations and of the breakdown of species boundaries through hybridization, he was nevertheless unable to take that small, but crucial, final step towards accepting the biological species. A consequence was that the taxonomy of "*Anoptichthys*" was fiercely contested by him not on its scientific merits but using metaphysical stances and personal criticism of colleagues who had come to a different conclusion.

This case represents an example of the truism that science is a human endeavor whose practitioners can have great difficulty in separating preconceptions and personal biases from the scientific consensus and the latest methodological approaches in the field. It is not only religious or social dogma that can hamper progress: the history of science has many examples of long-entrenched orthodoxies ultimately being swept away. As Carl Sagan (2011, p. 429) said, "*The cure for a fallacious argument is a better argument, not the suppression of ideas*".

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