RESEARCH ARTICLE

Vertical distribution of spiders (Araneae) in Central European shallow subterranean habitats

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Abstract

Shallow subterranean habitats are among the last habitats in Central Europe to be arachnologically researched. Using stratified pipe traps, we studied the vertical distribution of spiders in soil and interspaces in bedrock (shallow subterranean habitats). Specifically, we sampled fauna in different substrates, including limestone, sandy marlstone, sandy marl, claystone, loess, and artificial gravel accumulation. Employing stratified pipe traps allowed us to identify the depth at which particular species occurred. Across multiple years and sampling sites, we collected 76 spider species, 21 of which showed an affinity for subterranean microhabitats. Some of these species occurred in interspaces in soil and bedrock, whereas others have been previously found in subterranean ant nests and animal burrows. We collected five species (*Iberina microphthalma*, *Centromerus* cf. *piccolo*, *Porrhomma cambridgei*, *P. microcavense*, and *P. microps*) almost exclusively at depths over half a meter, suggesting the strong affinity of these species for a subterranean lifestyle. We provide diagrams of these species' vertical distribution and photo-document eye reduction. Our study demonstrates that poorly studied shallow subterranean habitats harbor diverse subterranean spider fauna, including several previously considered rare species in Central Europe.

Keywords

Introduction

Subterranean habitats range from large spaces in caves to tiny spaces such as fissure networks in bedrock or soil pores (Culver and Pipan 2014). They are typically characterized by the absence of light, almost 100% relative air humidity, and small temperature fluctuations over a year (Badino 2010). Animals fully adapted to subterranean habitats, e.g., those demonstrating depigmentation, reduced eyes, and other so-called troglomorphic traits, are sometimes caught by pitfall traps also on the surface but usually as singletons and only during the humid and cold season (Czech Arachnological Society 2022). Some subterranean habitats in central Europe are already well-studied from an arachnological point of view, especially caves or scree slopes (Růžička et al. 1995; Růžička et al. 2012). Caves and scree slopes provide relatively large spaces and thus can be inhabited by troglomorphic spiders like *Bathyphantes eumenis buchari* Růžička, 1988 (Růžička 1988) and *Meta menardi* (Latreille, 1804) (Mammola and Isaia 2014). In contrast, the arachnofauna of shallow subterranean habitats within coarse substrata, which are difficult to access, is poorly studied (Mammola et al. 2016). Shallow subterranean habitats are areas of habitable space that are less than 10 m in depth from the surface. These range from large areas such as shallow caves and lava tubes, to tiny areas such as cracks in ceilings, or spaces in soil (Culver and Pipan 2014). Different authors have used perforated pipe traps with one cup on the bottom to sample invertebrates in shallow subterranean habitats and deep soil strata (e.g., López and Oromí 2010; Deltshev et al. 2011; Jiménez-Valverde et al. 2015; Mammola et al. 2017; Ledesma et al. 2020). Conversely, Schlick-Steiner and Steiner (2000) constructed a trap consisting of a perforated pipe and a set of removable plastic cups situated on a central metal axis. The cups collect animals entering the pipe through holes at different depths, allowing to study the vertical distribution of arthropods in shallow subterranean habitats. For example, this trap design has been used to study the vertical distribution of spiders in soil and fissured rock (Laška et al. 2011; Růžička and Dolanský 2016), and springtails (Rendoš et al. 2016; Jureková et al. 2021), and myriapods (Haľková et al. 2020) in forested scree slopes.

In this study, we employed stratified pipe traps to explore spider fauna in soils, gravel, and loess (up to two meters in depth) on bedrock known to harbor fissure networks. The study aimed to describe the vertical distribution of spiders in these unique subterranean ecosystems and to describe species' preferences for certain types of substrates (microhabitats).

Materials and methods

Study sites

The research was carried out at ten sites in Czechia (Table 1, Fig. 1). In one site, Suchomasty, we documented temperature fluctuations measured by dataloggers Tinytag Ultra2 every three hours at the maximum sampling depth and on the surface (Fig. 5).

Figure 1. Locations of the study sites in Czechia. 1 – Kostelní Bříza, 2 – Raná, 3 – Suchomasty, 4 – Nebušice, 5 – Ctiněves, 6 – Hostovice, 7 – Mravín, 8 – Pouzdřany, 9 – Dolní Věstonice, 10 – Kurovice. For details, see Table 1.

Table 1. Characteristics of the study sites. n – number of pipe traps.

| Site	Coordinates	Altitude [m a.s.l.]/average annual temperature	n/pipe length	Years	Substrate	Vegetation
Kostelní Bříza	50.1119°N, 12.6349°E	570/7 °C	2/110 and 80 cm	2017–2021	gravel accumulation from a metal mine	sparse *Pinus sylvestris* and *Betula pendula* wood
Raná	50.4067°N, 13.7450°E	250/9 °C	1/110 cm	2014–2016	stony rendzina soil and fissure network in sandy marlstone	steppe grassland
Suchomasty	49.9062°N, 14.0667°E	405/8 °C	3/200 cm	2015–2020	Stony brown soil on limestone bedrock	*Carpinion* forest
Nebušice	50.1019°N, 14.3050°E	350/9 °C	1/110 cm	2017–2021	stony brown soil and fissure network in sandy marlstone (Fig. 2B)	*Carpinion* forest
Ctiněves	50.3707°N, 14.3180°E	265/9°C	2/120 cm	2019–2021	thin ranker soil on sandy gravel	planted *Robinia* wood
Hostovice	50.0093°N, 15.8509°E	230/9°C	1/130 cm	2015–2018	Stony scree soil on claystone bedrock (Fig. 2C)	deciduous bush
Mravín	49.9445°N, 16.0516°E	315/9°C	2/120 cm	2013–2021	thin rendzina soil on sandy marl	oak forest
Pouzdřany	48.9429°N, 16.6430°E	290/10 °C	2/90 cm	2017–2021	Thin rendzina on loess	steppe grassland
Dolní Věstonice	48.8856°N, 16.6562°E	205/10 °C	1/90 cm	2019–2022	bare loess	deciduous bush
Kurovice	49.2736°N, 17.5249°E	305/9°C	2/100 cm	2013–2021	Thin brown soil on limestone bedrock	*Carpinion* forest
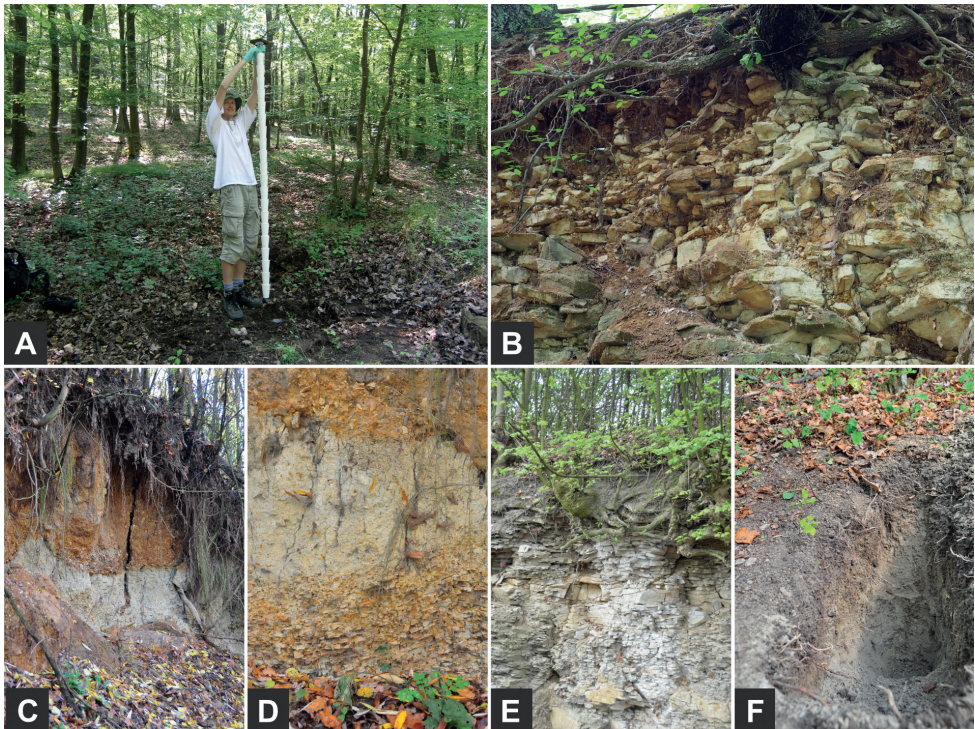
Figure 2. A a set of plastic cups taken from the pipe trap in the *Carpinus* forest on limestone near Suchomasty (photo M. Šafra); the soil profile is inhabited by *Porrhomma cambridgei*, see Fig. 3A **B** a fissure network in sandy marl near Nebušice, which was rich in *Porrhomma microcavense*, see Fig. 3D (the pipe trap was ten meters from this quarry wall) (photo M. Řezáč) **C, D** a scree soil near Hostovice, which was rich in *Porrhomma microps*, see Fig. 3E (photo J. Dolanský) **E** a fissure network in sandy marl near Mravín (the pipe trap was 200 meters from this profile) (photo J. Dolanský) **F** a hole for a pipe trap near Mravín in the soil, which was rich in *Iberina microphthalma* and *P. cambridgei*, see Fig. 3C (photo J. Dolanský).

Sampling

The plastic pipes (for lengths see Table 1) had an inner diameter of 7 cm and were perforated by oblique, 5 mm wide cuts. Plastic cups were mounted onto a threaded metal rod at 10 cm distances (for example, a 2-m long pipe contained 20 cups) (Fig. 2A) and contained a solution of 6% formaldehyde with few drops of detergent (for more details see Růžička and Dolanský 2016). We installed these traps in excavated holes or boreholes (a drilling machine was used in Suchomasty) and emptied them once a year. When we installed multiple pipes per site, each pipe was buried in a separate hole. Since traps were buried for long periods (between 2 and 8 years depending on the site), we expect the initial mixing of the soil layers to exert a minor role in the distribution of fauna.

Spider identification

Spiders were sorted and examined under an Olympus SZX12 stereomicroscope in 80% ethanol. Spider identification follows Nentwig et al. (2022), and nomenclature is in accordance with the World Spider Catalog (2022). The individuals were deposited at the institutions of the authors. Photographs were taken with an Olympus C-5060 wide-zoom digital camera mounted on an Olympus BX40 microscope. The images were processed using CombineZP image stacking software.

Relation to subterranean habitats

We classified the affinity of particular species to subterranean environments according to their vertical distribution. Further, we considered (1) morphological adaptations for subterranean life, (2) habitat, where they were found so far, (3) pattern of their distribution (common or rare on the surface habitats), (4) what environment they need for their lifestyle (see column "relation to the subterranean habitat – RSH" in Table 2). Morphological features, in particular depigmentation and a reduction in eye size, were recorded during identification, and the carapace size data were taken from the literature (Merrett et al. 1993; Weiss 1996; Růžička 2018, 2022; Šestáková et al. 2018; Nentwig et al. 2022). Data on spider occurrence in Czechia, habitat, and natural history were taken from a database of records of spiders in Czechia (Czech Arachnological Society 2022) and Buchar and Růžička (2002). The following categories were distinguished:

1 surface species occur only at the surface, on the ground, or in the vegetation. Their presence underground is accidental; they possess eyes of normal size and are normally pigmented.

2 detritus layer species live in detritus on the soil surface. They are rare in deeper soil layers. They possess eyes of normal size and are normally pigmented. Two special cases are within this category:

2B species with burrows in soil spend at least part of their lives in shallow burrows in soil; they possess eyes of normal size and are normally pigmented.

2H species requiring high air humidity require habitats with constantly high air humidity, regardless of whether the habitats are superficial or subterranean; they possess eyes of normal size and are normally pigmented.

3 soil species are more common deeper in humus soil than in detritus, but they appear on the soil surface relatively often; they are usually tiny and less pigmented, and their eyes are relatively smaller than those of their relatives. A special case is within this category:

3A soil ant nest species spend the majority of their lives in ant nests in the soil; they are less pigmented, and their eyes are relatively smaller than those of their surface relatives.

4   soil-bedrock species live in the basal mineral layers of soil on eroded bedrock surfaces (or in other spacious subterranean habitats) and appear on the soil surface very rarely; they are pale, and their eyes are significantly reduced; they can be larger with relatively long legs.

Data analyses

We used descriptive statistics [mean, standard error (SEM), and 95% confidence interval] to assess the species' preference for surface versus subterranean habitats, approximated via the depth of capture of each individual. In cases where the whole confidence interval was below a depth of 20 cm (the depth of the two uppermost cups in the trap, where the surface species can easily enter), we assumed the species to be more common below 20 cm depth than above it (the species shown in bold in Table 2). Data were handled in R, version 3.6.2 (R Core Team 2019).

Results

We captured 1179 adult spider specimens belonging to 76 species. In total, 17 species were found to be more common under the surface than on the surface (shown in bold in Table 2); singletons were not evaluated, but they are included in Table 2. Five species occurred preferentially below 70 cm (more than 15 specimens, Fig. 3). These were *Porrhomma cambridgei*, *Porrhomma microps*, *Porrhomma microcavense*, *Iberina microphthalma*, and *Centromerus* cf. *piccolo.* They possessed remarkably reduced eyes (Fig. 4). We called them "soil-bedrock species", as they probably occur in the interface between the soil and bedrock. In addition to these five species, we also considered *Pseudomaro aenigmaticus*, *Nesticus cellulanus*, *Jacksonella falconeri*, *Palliduphantes insignis*, *Centromerus* cf. *minutissimus* and *Theonoe minutissima* as belonging to this category. We further considered eight species to represent soil species (see the chapter Relation to subterranean habitats in MM for definitions of these categories: *Mioxena blanda*, *Porrhomma microphthalmum*, *Cicurina cicur*, *Tapinocyboides pygmaeus*, *Centromerus cavernarum*, *Centromerus serratus*, *Palliduphantes alutacius*, and *Palliduphantes pallidus*. We also recorded two myrmecophilous species occurring in ant nests in the soil, *Mastigusa arietina*, and *Syedra myrmicarum*. The remaining captured species were classified as detritus layer species (28) or as species with no relation to subterranean habitats (27).

Discussion

At least 21 species collected in our pipe traps exhibited morphological (eye reduction and depigmentation) and distributional (rare occurrence in surface habitats and regular presence in subterranean habitats) characteristics typical of subterranean fauna (categories 3 and 4 in Table 2). Of these, twelve species were more frequent below 20 cm than on the surface (marked bold in Table 2). The numbers of collected individuals in the remain-

Table 2. Vertical distribution of the collected spider species sorted according to the average depth from the deepest point toward the surface. Depth: mean ± SEM (95% confidence interval). The species that were significantly more common in deep subterranean habitats than close to the surface are shown in bold. n – number of specimens captured. Sites: Bří – Kostelní Bříza, Cti – Ctiněves, Hos – Hostovice, Kur – Kurovice, Mra – Mravín, Neb – Nebušice, Pou – Pouzdřany, Ran – Raná, Such – Suchomasty, Věs – Dolní Věstonice. RSH – relation to the subterranean habitat: 1 – surface species; 2 – detritus layer species (B – with burrows in the soil, H – requiring high air humidity); 3 – soil species (below the detritus layer, but found relatively often on the soil surface, A – ant nest species); 4 – soil-bedrock species (basal layers of the soil, very rarely found on the soil surface).

Species	Family	Depth [cm]	n	Geology	Site	RSH
Porrhomma cambridgei Merrett, 1994	Linyphiidae	100 ± 5.9 (89–112)	45	sandy marl, limestone	Mra, Such	4
Porrhomma microps (Roewer, 1931)	Linyphiidae	93 ± 2.0 (89–97)	156	sandy marl, limestone, claystone	Hos, Kur, Mra, Such	4
Porrhomma microcavense Wunderlich, 1990	Linyphiidae	86 ± 3.3 (80–93)	24	sandy marlstone	Neb	4
Mastigusa arietina (Thorell, 1871)	Dictynidae	80 ± 21.2 (38–122)	2	sandy marl, gravel heap	Bří, Mra	3A
Iberina microphthalma (Snazell & Duffey, 1980)	Hahniidae	78 ± 3.7 (70–85)	25	sandy marl	Mra	4
Centromerus cf. **piccolo** Weiss, 1996	Linyphiidae	75 ± 3.5 (68–82)	17	loess	Pou, Věs	4
Porrhomma campbelli F. O. Pickard-Cambridge, 1894	Linyphiidae	69 ± 2.3 (65–74)	12	gravel heap	Bří	2
Pseudomaro aenigmaticus Denis, 1966	Linyphiidae	65 ± 31.8 (3–127)	2	gravel heap	Bří	4
Walckenaeria capito (Westring, 1861)	Linyphiidae	63 ± 8.0 (47–78)	8	sandy marlstone, gravel heap	Bří, Ran	2
Mioxena blanda (Simon, 1884)	Linyphiidae	60 ± 23.0 (15–105)	3	sandy marl and marlstone, gravel	Cti, Mra, Ran	3
Nesticus cellulanus (Clerck, 1757)	Nesticidae	50 ± 0 (50)	1	claystone	Hos	4
Robertus neglectus (O. Pickard-Cambridge, 1871)	Theridiidae	50 ± 11.5 (27–73)	3	limestone	Such	2
Porrhomma microphthalmum (O. Pickard-Cambridge, 1871)	Linyphiidae	43 ± 9.0 (26–61)	3	sandy marl, limestone, claystone	Hos, Mra, Such	3
Cicurina cicur (Fabricius, 1793)	Dictynidae	42 ± 1.5 (39–45)	285	loess, sandy marl, marlstone, limestone, claystone, gravel heap	Bří, Hos, Kur, Mra, Neb, Ran, Such, Věs	3
Harpactea lepida (C. L. Koch, 1838)	Dysderidae	40 ± 5.4 (29–51)	7	limestone, gravel heap	Bří, Such	2H
Centromerus cavernarum (L. Koch, 1872)	Linyphiidae	40 ± 14.1 (12–68)	2	gravel heap	Bří	3
Jacksonella falconeri (Jackson, 1908)	Linyphiidae	40 ± 0 (40)	1	gravel heap	Bří	4
Coelotes terrestris (Wider, 1834)	Agelenidae	40 ± 0 (40)	1	sandy marl	Mra	2B
Micrargus herbigradus (Blackwall, 1854)	Linyphiidae	39 ± 3.8 (31–46)	25	sandy marl and marlstone, limestone, gravel heap	Bří, Kur, Mra, Neb	2
Palliduphantes insignis (O. Pickard-Cambridge, 1913)	Linyphiidae	35 ± 4.0 (27–43)	31	loess	Pou, Věs	4
Syedra myrmicarum (Kulczyński, 1882)	Linyphiidae	35 ± 3.5 (28–42)	2	sandy marl	Mra	3A
Palliduphantes alutacius (Simon, 1884)	Linyphiidae	34 ± 2.2 (30–38)	83	loess, sandy marl, claystone, limestone	Hos, Kur, Mra, Věs	3
Palliduphantes pallidus (O. Pickard-Cambridge, 1871)	Linyphiidae	33 ± 3.9 (26–41)	35	sandy marlstone, gravel heap	Bří, Neb, Ran	3
Centromerus cf. *minutissimus* Merrett & Powel, 1993	Linyphiidae	30 ± 0 (30)	1	gravel heap	Bří	4

Species	Family	Depth [cm]	n	Geology	Site	RSH
Metopobactrus prominulus (O. Pickard-Cambridge, 1873)	Linyphiidae	30 ± 0 (30)	1	gravel heap	Bří	2
Walckenaeria dysderoides (Wider, 1834)	Linyphiidae	30 ± 6.2 (18–42)	8	loess, limestone	Kur, Such, Věs	2
Walckenaeria obtusa Blackwall, 1836	Linyphiidae	30 ± 0 (30)	1	limestone	Kur	2
Robertus arundineti (O. Pickard-Cambridge, 1871)	Theridiidae	30 ± 0 (30)	1	limestone	Such	2
Piratula uliginosa (Thorell, 1856)	Lycosidae	30 ± 7.1 (16–44)	2	gravel heap	Bří	1
Clubiona terrestris Westring, 1851	Clubionidae	30 ± 7.1 (16–44)	2	sandy marl, claystone	Hos, Mra	1
Robertus lividus (Blackwall, 1836)	Theridiidae	26 ± 3.2 (19–32)	18	loess, limestone	Kur, Pou, Věs	2
Agyneta rurestris (C. L. Koch, 1836)	Linyphiidae	25 ± 3.5 (18–32)	2	limestone, gravel heap	Bří, Such	1
Histopona torpida (C. L. Koch, 1837)	Agelenidae	25 ± 10.6 (4–46)	2	sandy marl	Mra	2H
Hahnia pusilla C. L. Koch, 1841	Hahniidae	24 ± 2.1 (20–28)	16	sandy marl, limestone, gravel heap	Bří, Kur, Mra	2
Harpactea rubicunda (C. L. Koch, 1838)	Dysderidae	23 ± 2.7 (18–29)	6	sandy marl and marlstone, limestone	Mra, Neb, Such	2
Theonoe minutissima (O. Pickard-Cambridge, 1879)	Theridiidae	23 ± 2.7 (18–29)	6	gravel heap	Bří	4
Dysdera cechica Řezáč, 2018	Dysderidae	23 ± 3.7 (16–30)	10	loess, limestone	Kur, Pou, Věs	2
Diplostyla concolor (Wider, 1834)	Linyphiidae	22 ± 1.4 (20–25)	102	claystone, sandy marlstone, limestone	Hos, Kur, Neb, Ran	2
Dysdera erythrina (Walckenaer, 1802)	Dysderidae	20 ± 2.2 (16–24)	11	limestone, gravel heap	Bří, Such	2
Episinus truncatus Latreille, 1809	Theridiidae	20 ± 7.7 (5–35)	3	loess	Pou	1
Mermessus trilobatus (Emerton, 1882)	Linyphiidae	20 ± 0 (20)	1	claystone	Hos	1
Tapinocyba insecta (L. Koch, 1869)	Linyphiidae	20 ± 0 (20)	1	gravel	Cti	2
Tapinocyboides pygmaeus (Menge, 1869)	Linyphiidae	20 ± 0 (20)	1	gravel heap	Bří	3
Amaurobius jugorum L. Koch, 1868	Amaurobiidae	20 ± 0 (20)	1	sandy marl	Mra	2B
Trochosa ruricola (De Geer, 1778)	Lycosidae	20 ± 7.7 (5–35)	3	sandy marlstone	Ran	2B
Phrurolithus festivus (C. L. Koch, 1835)	Phrurolithidae	18 ± 1.7 (15–22)	31	loess, sandy marl and marlstone, limestone, gravel heap, gravel	Bří, Cti, Mra, Neb, Ran, Such, Věs	1
Centromerus sylvaticus (Blackwall, 1841)	Linyphiidae	17 ± 1.8 (13–20)	13	loess, limestone, sandy marlstone, gravel heap	Bří, Neb, Ran, Such, Věs	2
Trachyzelotes pedestris (C. L. Koch, 1837)	Gnaphosidae	17 ± 1.2 (14–19)	18	loess, sandy marlstone, limestone, gravel	Cti, Kur, Ran, Věs	1
Inermocoelotes inermis (L. Koch, 1855)	Agelenidae	16 ± 3.2 (10–22)	5	limestone, gravel heap	Bří, Kur	2B
Haplodrassus silvestris (Blackwall, 1833)	Gnaphosidae	15 ± 3.5 (8–22)	2	limestone	Kur	1
Centromerus serratus (O. Pickard-Cambridge, 1875)	Linyphiidae	14 ± 2.1 (10–18)	5	sandy marl	Mra	3
Agroeca cuprea Menge, 1873	Liocranidae	13 ± 1.4 (8–32)	3	sandy marlstone, loess	Pou, Ran	1
Apostenus fuscus Westring, 1851	Liocranidae	13 ± 0.7 (11–14)	31	limestone	Kur	1
Tenuiphantes flavipes (Blackwall, 1854)	Linyphiidae	11 ± 0.5 (10–12)	11	loess, limestone	Kur, Such, Věs	1
Euryopis flavomaculata (C. L. Koch, 1836)	Theridiidae	10 ± 0 (10)	2	limestone	Kur	2

Species	Family	Depth [cm]	n	Geology	Site	RSH
Agyneta saxatilis (Blackwall, 1844)	Linyphiidae	10 ± 0 (10)	1	loess	Pou	1
Linyphia hortensis Sundevall, 1830	Linyphiidae	10 ± 0 (10)	1	sandy marl	Mra	1
Micrargus subaequalis (Westring, 1851)	Linyphiidae	10 ± 0 (10)	1	sandy marlstone	Ran	2
Oedothorax retusus (Westring, 1851)	Linyphiidae	10 ± 0 (10)	1	limestone	Kur	2
Pelecopsis radicicola (L. Koch, 1872)	Linyphiidae	10 ± 0 (10)	1	gravel heap	Bří	2
Aulonia albimana (Walckenaer, 1805)	Lycosidae	10 ± 0 (10)	1	limestone	Kur	2B
Pardosa lugubris (Walckenaer, 1802)	Lycosidae	10 ± 0 (10)	45	sandy marlstone, limestone	Kur, Neb	1
Pardosa riparia (C. L. Koch, 1833)	Lycosidae	10 ± 0 (10)	5	sandy marlstone	Ran	1
Trochosa terricola Thorell, 1856	Lycosidae	10 ± 0 (10)	2	limestone	Such	2B
Zora spinimana (Sundevall, 1833)	Miturgidae	10 ± 0 (10)	1	limestone	Kur	1
Agroeca brunnea (Blackwall, 1833)	Liocranidae	10 ± 0 (10)	1	limestone	Kur	1
Phrurolithus pullatus (C. L. Koch, 1835)	Phrurolithidae	10 ± 0 (10)	1	loess	Pou	1
Zodarion germanicum (C. L. Koch, 1837)	Zodariidae	10 ± 0 (10)	5	limestone	Kur	1
Drassyllus praeficus (L. Koch, 1866)	Gnaphosidae	10 ± 0 (10)	1	loess	Pou	1
Drassyllus pumilus (C. L. Koch, 1839)	Gnaphosidae	10 ± 0 (10)	4	loess	Pou	1
Haplodrassus umbratilis (L. Koch, 1866)	Gnaphosidae	10 ± 0 (10)	3	gravel heap	Bří	1
Zelotes apricorum (L. Koch, 1876)	Gnaphosidae	10 ± 0 (10)	8	limestone	Kur	1
Ozyptila praticola (C. L. Koch, 1837)	Thomisidae	10 ± 0 (10)	1	limestone	Kur	1
Ozyptila trux (Blackwall, 1846)	Thomisidae	10 ± 0 (10)	1	loess	Pou	1
Xysticus luctator L. Koch, 1870	Thomisidae	10 ± 0 (10)	1	limestone	Kur	1
Euophrys frontalis (Walckenaer, 1802)	Salticidae	10 ± 0 (10)	2	limestone	Kur	1

ing nine species were too low to demonstrate the same preference. On the other hand, five other species, *Porrhomma campbelli*, *Walckenaeria capito*, *Robertus neglectus*, *Harpactea lepida*, and *Micrargus herbigradus*, did not exhibit morphological or distributional features (they are common in surface habitats) characteristic of subterranean fauna but were more frequent below 20 cm than on the surface (also marked bold in Table 2). However, this result is based on low specimen numbers. The remaining species did not have any affinity for the subterranean environment; they usually occur on the soil surface or in detritus. These species probably crawled down the substrate along the pipe where the soil became loose due to digging. Such vertical migration could have happened during autumn and winter months when even the surface species search for suitable spots for overwintering.

The main subterranean spider groups

The captured subterranean species belonged to the families Linyphiidae, Hahniidae, Theridiidae, and Nesticidae, with Linyphiidae having the greatest abundance and species richness. Three genera dominate in the Central European shallow subterranean habitats: *Centromerus*, *Palliduphantes*, and especially *Porrhomma*. At two study sites, Mravín and Suchomasty, *Porrhomma cambridgei* and *Porrhomma microps* were found to occur syntopically. In both cases, the smaller *P. cambridgei* was found deeper than the larger *P. microps* (Fig. 3A, C). So far, *Porrhomma campbelli* has been known from the moss in wet habitats, such as peat bogs, spruce forests, and brook and pond margins (Růžička 2018). Here, we present the first finding of this species from subterranean habitats in Czechia.

The genus *Centromerus* was represented by four species, two of which, *C.* cf. *piccolo* and *C.* cf. *minutissimus*, were new to Czechia. We captured 17 specimens of *Centromerus* cf. *piccolo* at depths of 50–90 cm in loess accumulations in south Moravia. Previously, *Centromerus piccolo* had been known only from Germany (Weiss 1996; Nentwig et al. 2022). As the genitalic morphology of our specimens slightly differs from the illustrated type specimens of *C. piccolo* (Weiss 1996), we can not exclude the possibility that this is a closely related new species. In the artificial gravel accumulation in western Bohemia, we found two *Centromerus* females
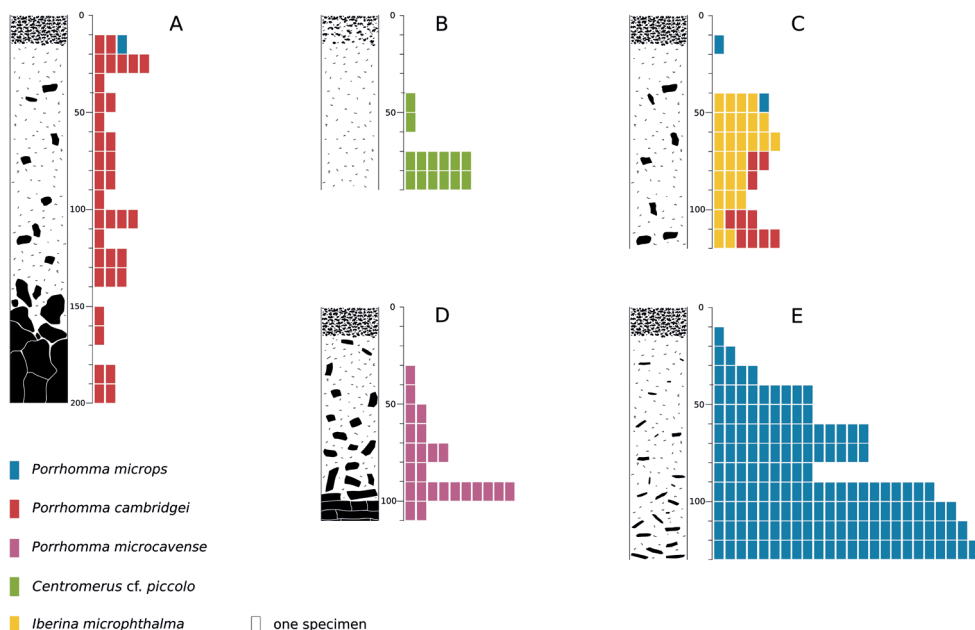

Figure 3. Panels show soil profiles and the depth-dependent occurrence of microphthalmic spider species (depths in cm) **A** Suchomasty, soil on limestone **B** Věstonice, loess **C** Mravín, stony soil above sandy marl **D** Nebušice, stony soil, and fissure network in sandy marl **E** Hostovice, stony soil above claystone.

Figure 4. Carapaces of female spider species found deep in the Czech shallow subterranean habitats **A** *Porrhomma microps* from Hostovice **B** *P. microcavense* from Nebušice **C** *P. cambridgei* from Suchomasty **D** *Iberina microphthalma* from Mravín **E** *Centromerus* cf. *piccolo* from Dolní Věstonice. Scale bars: 0.1 mm (photos V. Růžička).
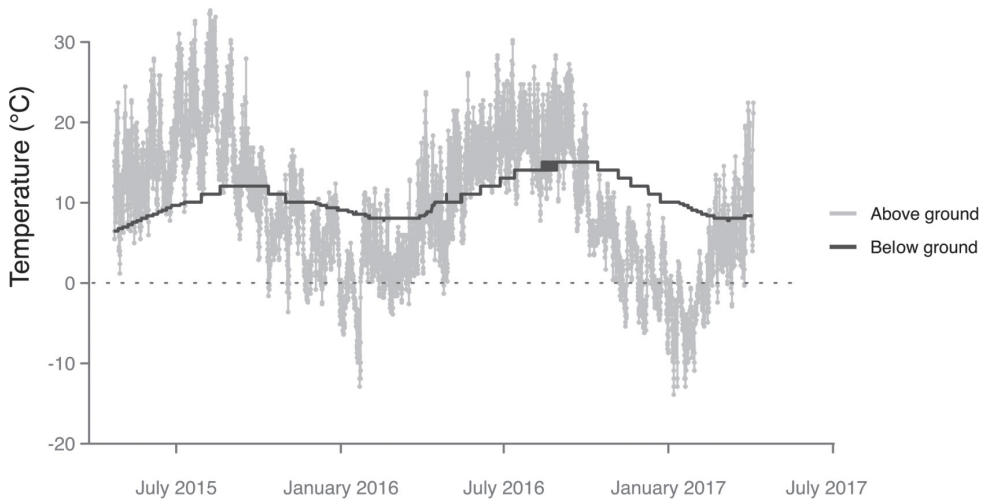

Figure 5. Temperature fluctuations in shallow subterranean habitats (below ground) were minor compared to temperature fluctuations on the surface (above ground). Data were collected from the study site at Suchomasty, between 23 April 2015 to 15 April 2017, taking one measurement every three hours. Above ground: 2.5 m above the ground, in shade. Below ground: at 2 m depth.

that are not described in the literature. Based on somatic characters (including small body size, eye reduction, and depigmentation) and the fact that the only Central European *Centromerus* species with unknown females was *Centromerus minutissimus*, which were known from England, Germany (the site in southern Saxony-Anhalt is only 200 km northwest of our site), south France and Spain (Merrett et al. 1993; Cárdenas and Barrientos 2011; Nentwig et al. 2022, GBIF – www. gbif.org, INPN – www.inpn.mnhn.fr), we preliminarily determined these individuals to be *Centromerus* cf. *minutissimus*.

We captured three species of the genus *Palliduphantes*. *Palliduphantes insignis* has been found in Czechia very rarely, only on the steppes on rocks or loess in warm areas

(Buchar and Růžička 2002). We found 31 specimens at a depth of approximately 30 cm in loess. Thus, its preferred microhabitats are probably micro spaces in soil and fissures in bedrock. *Palliduphantes pallidus* and *Palliduphantes alutacius* sensu Miller and Obrtel (1975) are closely related taxa that are not morphologically clearly separated in Czechia. Instead, there are mainly typical *Palliduphantes pallidus* in the western part of Czechia, while in the eastern part typical *Palliduphantes alutacius* occurs, and morphologically intermediate forms can be found between. This pattern probably demonstrates the introgression of these two taxa in this territory. These species are among the most common subterranean species in Czechia and are also often found at the surface.

Concerning the remaining subterranean linyphiid spiders, we found four species of the subfamily Erigoninae: *Jacksonella falconeri*, *Mioxena blanda*, *Pseudomaro aenigmaticus*, and *Tapinocyboides pygmaeus*. Singletons of these species have rarely been found on the surface, usually in open xerothermic habitats (Czech Arachnological Society 2022). Our data show that the main microhabitats of these species are interspaces in various substrata.

The subterranean Hahniidae spiders were represented by three species. European records of *Iberina microphthalma* were summarized by Růžička and Dolanský (2016). Furthermore, in Czechia, this species was collected in the substratum on claystone, sandy marlstone, or sandy marl (Růžička 2022). We found 17 specimens in the soil on sandy marl at depths of 40–120 cm (Fig. 3C). *Cicurina cicur* is common under stones, in leaf litter, and decaying wood in forests, as well as in shallow subterranean habitats (Buchar and Růžička 2002; Růžička and Dolanský 2016). Because of its morphological appearance – depigmentation and small eyes – it is expected for this species to spend most of its life in the subterranean environment, especially in southern Europe (Mammola et al. 2022). Indeed, we found it to be dominant in the studied shallow subterranean habitats.

Nesticus cellulanus is the only representative of Nesticidae, the spiders mainly occurring in subterranean environments (Mammola et al. 2022). As this species is relatively large, it mainly occurs in caves, scree slopes, or man-made subterranean spaces, whereas it is rare in interspaces in the substratum.

The family Theridiidae was represented by *Theonoe minutissima* and some species of the genus *Robertus*. *Theonoe minutissima* regularly occurs in scree slopes, we found it only inside artificial gravel accumulation. *Robertus* species recorded during our study are common in detritus.

Myrmecophilous species

At two sites, Kostelní Bříza and Mravín, we captured the relatively large depigmented hahniid spider *Mastigusa arietina*, which is known to live in subterranean ant nests (Nentwig et al. 2022). At the Mravín site, it was found with another myrmecophilous spider, *Syedra myrmicarum*. The dominant co-occurring ant species was *Lasius flavus* (Fabricius, 1782).

Troglomorphism versus edaphomorphism

The body size and relative length of appendages of subterranean species may depend on the size of the spaces they inhabit (Pipan and Culver 2017). Predictably, an elongation

of appendages is an adaptation to life in relatively large subterranean spaces, typically caves (troglomorphism, Zacharda 1979). In contrast, a diminished body size represents an adaptation to life in relatively small, narrow subterranean spaces, typically of soils (edaphomorphism, Zacharda 1979). Altogether, the species collected during this study cover the continuum from troglomorphic to edaphomorphic species. For example, relatively large, long-legged *Porrhomma microps* (carapace width 0.84 mm) and *P. microcavense* (carapace width 0.75 mm) represent typical troglomorphic species. They are characteristic of the interface between soil and sandy marlstone that harbors relatively large interspaces (Fig. 2B). Because of harsh climatic conditions during Quaternary glaciations, Central Europe lacks large troglomorphic spiders, known, for example, from Mediterranean caves (Culver et al. 2006). The most troglomorphic species of this region are still small enough to also occur in soil (Růžička 1999).

The edaphomorphic species collected during this study were tiny *Centromerus* (*C.* cf. *piccolo* and *C.* cf. *minutissimus*), *Porrhomma cambridgei*, *Iberina microphthalma*, and tiny Erigoninae linyphiids, all of which have carapace widths less than 0.6 mm. They probably live in narrow interspaces in soil or loess; however, they also occur in deep caves (Růžička and Buchar 2008; Růžička 2018, 2022).

Preferences for bedrock and climate

Different shallow subterranean habitats presumably provide microhabitats that are preferred by different species. Indeed, some subterranean species collected in the soil-bedrock interface seem to exhibit a preference for specific depths and substrates. For example, *Centromerus* cf. *piccolo* is characteristic of loess, and *Porrhomma microcavense* and *Iberina microphthalma* are characteristic of sandy marlstone. The species that occur in the surface layer of the soil seem not to express such preferences (for example, *Centromerus serratus*, *C. cavernarum*, *Mioxena blanda*, *Palliduphantes* spp., *Porrhomma microphthalmum*, *P. microps*, *Tapinocyboides pygmaeus*, *Cicurina cicur*).

The fauna of shallow subterranean habitats seems to be richer in warm areas than in cold ones. The species *Centromerus* cf. *piccolo*, *Mioxena blanda*, *Palliduphantes insignis*, *Porrhomma cambridgei*, and *Iberina microphthalma* are restricted to the warmest regions of Czechia. Additionally, vegetation can be expected to modify the temperature regime in the soil. A large number of the soil species found during this study live in substrates that occur in partly open xerothermic habitats (for example, *Centromerus* cf. *piccolo*, *Palliduphantes insignis*, *Tapinocyboides pygmaeus*, and *Iberina microphthalma*). On the other hand, some species live in habitats that maintain stable humidity and temperature, such as scree forests (e.g., *Centromerus cavernarum*, *Porrhomma campbelli*, *Theonoe minutissima*).

Dispersal

Interestingly, the only artificial habitat that was studied, an almost hundred-year-old gravel heap from a polymetallic ore mine near Kostelní Bříza, harbors relatively rich subterranean fauna. Several species with relatively small eyes, in particular *Jacksonella*

falconeri, *Pseudomaro aenigmaticus*, *Tapinocyboides pygmaeus*, *Centromerus cavernarum*, *Centromerus* cf. *minutissimus*, *Palliduphantes pallidus*, *Porrhomma campbelli*, *Theonoe minutissima*, *Cicurina cicur*, and *Mastigusa arietina* were found here. Thus, at least these species are able to colonize new isolated sites, probably by ballooning, and they cannot be considered relics like the endemic fauna in limestone caves in the Mediterranean. For *Pseudomaro aenigmaticus*, this ability was confirmed by the capture of specimens in aeroplankton at 12 m height (Blick and Kreuels 2002).

Conclusion

Our study demonstrates that underexplored shallow subterranean habitats in Central Europe harbor rich subterranean spider fauna. Some of these species were considered very rare in the past. However, we came to the same conclusion as Polenec (1970): these species are, in fact, quite abundant in these hardly accessible and largely overlooked habitats.

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RESEARCH ARTICLE

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Two new sympatric cave species of *Castellanethes* (Isopoda, Oniscidea, Olibrinidae) from Western High Atlas of Morocco

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Abstract

Two new sympatric isopod species of the genus *Castellanethes* (Olibrinidae) are described from caves located in the Western High Atlas of Morocco. Both species present troglomorphic traits, such as the absence of body pigmentation and eyes and are, therefore, considered cave-dwelling species (troglobitic). *Castellenethes ougougensis* **sp. nov.** was found in five caves, while *C. ighousi* **sp. nov.** is an amphibious species found in only two caves, which also harbour populations of *C. ougougensis* **sp. nov.** Additionally, notes on their habitats are provided, as well as a discussion on their conservation status.

Keywords

Introduction

Globally, the terrestrial isopod richness is represented by 3,984 species (Campos-Filho and Taiti 2021). In Morocco, 81 species of Oniscidea have been recorded, belonging to 14 families: Ligiidae with one species in one genus (*Ligia*); Tylidae with one species in one genus (*Tylos*); Trichoniscidae, with six species in four genera (*Finaloniscus*, *Haplophthalmus* and *Graeconiscus* – one sp each, *Trichoniscus* – three spp); Olibrinidae with one species in one genus (*Castellanethes*); Detonidae with one species in one genus (*Armadilloniscus*); Halophilosciidae with two species in two genera (*Halophiloscia* and *Stenophiloscia* – one sp each); Philosciidae, with three species in three genera (*Anaphiloscia*, *Ctenoscia* and *Paractenoscia* – one sp each); Stenoniscidae with one species in one genus (*Stenoniscus*); Spelaeoniscidae, with nine species in four genera (*Atlantoniscus* and *Barbarosphoera* – one sp each, *Marghreboniscus* – two spp and *Spelaeoniscus* – five spp); Platyarthridae, with four species in two genera (*Platyarthrus* – three spp and *Trichorhina* – one sp); Bathytropidae with one species in one genus (*Bathytropa*); Armadillidae with one species in one genus (*Armadillo*); Porcellionidae, with 43 species in six genera (*Porcellio* – 25 spp, *Porcellionides* – nine spp, *Soteriscus* – four spp, *Agabiformius* – two spp, *Lucasius* – two spp and *Leptotrichus* – one sp); and Armadillidiidae, with seven species in two genera (*Armadillidium* – five spp, *Eluma* – two spp). (Schmalfuss 2003; Schmidt and Leistikow 2004; Montesanto et al. 2011; Taiti and Rossano 2015; Garcia et al. 2020).

Although biospeological investigations started in Morocco in the 19ᵗʰ century with the description of two cave beetles by Dieck (1869) (*Geocharis messiniana* and *Crypharis robusta*), only three troglobitic isopods have been recorded since then: the Olibrinid *Castellanethes soloisensis* (Vandel, 1959) from Goran Cave, located in Cape Beddouza near the City of Safi in Central Morocco, the Philoscid *Paractenoscia cavernicola* Taiti & Rossano, 2015 and the Trichoniscid *Graeconiscus gevi* Garcia, Miralles-Núñez & Pérez Fernández, 2020, both from Ghar Knadel Cave, located in the north of Morocco near Laou River.

Caves of the Tamri-Tamraght Basin in the Western High Atlas, were subject to several investigations for years in term of biodiversity studies. Therefore, several species from other groups have been described; the linyphiid spider *Lessertia barbara* (Simon, 1884), the dysderid spider *Dysdera drescoi* Ribera, 1983, the liocranid spider *Agraecina agadirensis* Lecigne, Lips, Moutaouakil & Oger, 2020, the pholcid spider *Maghreba kahfa* Huber, 2022, the carabid beetles *Antoinella fadriquei* Mateu & Escolà, 2006 and *Siagona taggadertensis* Juger & Faille, 2011, the staphylinid beetles *Domene cantonsi* Espanol, 1972 and *Apteranillus bichaini* Perreau & Faille, 2012 and the paradoxosomatid millipede *Jeekelosoma heptarachne* Enghoff & Reboleira, 2019. However, no cave-dwelling isopod has been described to date from this region.

The following work provides a description of two new species of troglobitic Oniscidea from the Western High Atlas (Tamri-Tamraght Basin, Agadir Ida Outanane area), both belonging to the genus *Castellanethes* Brian, 1952. The two species described herein are the first cave-restricted isopods described for this region. In addition, notes on their habitats are provided, along with a discussion on their conservation status.

Materials and methods

Seven limestone caves located in the Tamri Basin (Western High Atlas) were sampled, five of which are located near the Talmat River: the Chauve-souris Cave (30°36'46.40"N, 9°28'01.63"W), the Imi Ougoug Cave (30°36'44.24"N, 9°28'01.56"W), the Ghar Sghir Cave (30°36'35.39"N, 9°28'09.26"W), the Ifri N'Tyaline Cave named also Ifri N'Talmat Cave (30°35'59.20"N, 9°28'56.78"W) and the Ifri Taghrart Ounakrim Cave (30°35'29.44"N, 9°30'56.95"W). The two other localities are the Win Timdouine Cave (30°40'49.75"N, 9°20'40.66"W) and the Tigmi N'Dou Akkal Cave (30°45'34.60"N, 9°25'06.96"W) (Fig. 1).

Specimens were collected with a paintbrush and preserved in 96% ethanol. The collected specimens were dissected in the laboratory for morphological observations. The dissected parts were clarified by a solution of lactic acid and mounted on slides using Hoyer's solution (Anderson 1954). Drawings were performed using a *camera lucida* coupled with an Olympus CHA microscope. Some specimens were dried in an alcoholic series for the analysis of morphological structures using the scanning electron microscope Hitachi TM4000. The type material is deposited in the Biospeological collection of the Natural History Museum of Marrakech, Cadi Ayyad University, Morocco.
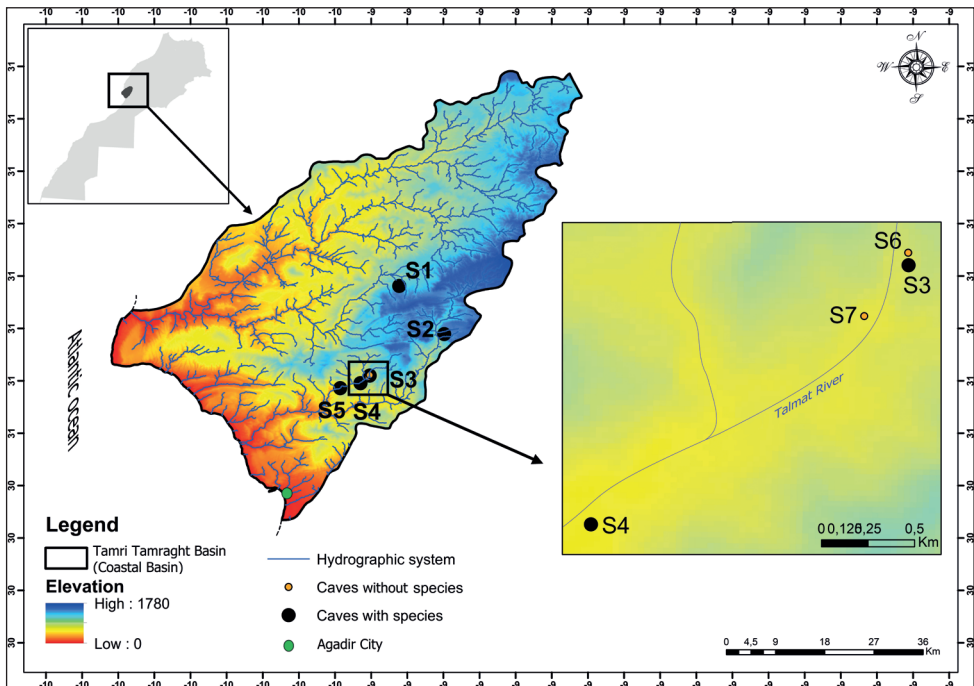

Figure 1. Location of the seven caves prospected for the study of troglobitic isopods in the Tamri Basin; (S1) Tigmi N'Dou Akkal; (S2) Win Timdouine; (S3) Imi Ougoug; (S4) Ifri N'Tyaline; (S5) Taghrart Ounakrim; (S6) Chauve-souris Cave; (S7) Ghar Sghir.

Physico-chemical parameters were measured using a HI98194 portable probe and a HI9564 portable thermohygrometer. The temperature and humidity of the entrance of the cave and the right strand were measured on 20/04/2022. Water temperature, dissolved oxygen and conductivity were measured on 20/04/2016 and on 20/04/2022.

Results

Troglobitic Oniscidea were only found in five caves: Imi Ougoug, Ifri N'Tyaline, Win Timdouine Cave, Taghrart Ounakrim Cave and Tigmi N'Dou Akkal Cave. Hence, no specimens were observed in Chauves-souris and Ghar Sghir Caves (Fig. 1).

Taxonomy

Family Olibrinidae Budde-Lund, 1913
Genus *Castellanethes* Brian, 1952

Remarks. This genus includes five known species: *C. sanfilippoi* Brian, 1952 from Italy; *C. velox* (Vandel, 1955) from Lebanon; *C. soloisensis* (Vandel, 1959) from a Moroccan cave (Goran Cave); *C. insularis* (Taiti, Ferrara & Kwon, 1992) from Indonesia (Togian Islands) and recorded also from the coast of Kenya (Tabasso, near Malindi) by Taiti and Ferrara (2004); and *C. fluviatilis* (Taiti & Ferrara, 2004) from Yemen (Socotra Island).

Castellanethes soloisensis (Vandel, 1959)
Figs 8A, D, 9A, B, 10A, D, G, 11A–C

Trichoniscus soloisensis Vandel, 1959: 162, fig. 3; Cruz 1991: 98, tab.1 (partim); Schmalfuss 2003: 313.
Adoniscus soloisensis: Taiti and Ferrara 2004: 227; Taiti and Gardini 2022: 97, 103, figs IV–V.

Material examined. Morocco • 3 ♂, 2 ♀ (mounted on a slide), Safi-Goran Cave, 32°33'18.17"N, 9°15'09.49"W, alt. 36 m, 14 Dec 2019, Moutaouakil S. leg., MHNM ZAI15.

Remarks. *Castellanethes soloisensis* is the first cave-dwelling isopod described from Morocco. This species was described by Vandel (1959) on the basis of 1 ♂ and 13 ♀ from the Goran Cave. Recently, the species was transferred to the genus *Castellanethes* by Taiti and Gardini (2022), based on the characters present in a female syntype from the same cave. So far, *C. soloisensis* seems to be endemic to the karst area near the City of Safi. Cruz (1991) recorded this species from several caves in Morocco: four caves near the city of Azilal-Central High Atlas (Ifri N'Touaya Cave, Ifri Bernat Cave, Ifri Bouylg-Maine and Sima Aurat Cave), one cave near the City of Agadir-Western High

Atlas (Wit Tamdoun which corresponds to Win Timdouine Cave) and Goran Cave. Most probably, the specimens from caves of the Central High Atlas do not belong to *C. soloisensis*, while specimens from Win Timdouine Cave probably correspond to *C. ougougensis* sp. nov. described below. Re-examination of the collection studied by Cruz (1991) is needed for a correct identification.

Castellanethes ougougensis Moutaouakil & Boulanouar, sp. nov.

https://zoobank.org/B2EE7107-4D7B-46C2-99C5-1668C3688183
Figs 2–4, 8B, E, 9C, D, 10B, E, H, 11D–F, 12H

Material examined. *Holotype*: Morocco • 1 ♂ (mounted on a slide), Agadir-Imi Ougoug Cave, alt. 773 m, 29 Dec 2019, Moutaouakil S. leg. MHNM ZAI01. *Paratypes*: Morocco • 2 ♀, Imi Ougoug Cave, 26 Feb 2020, Moutaouakil S. leg., MHNM ZAI03 and MHNM ZAI04 • 1 ♂, Ifri N'Tyaline Cave, 27 Oct 2019, Lips J. leg., MHNM ZAI05 • 1 ♂, Win Timdouine Cave, 31 Oct 2019, Moutaouakil S. leg., ZAI06 • 1 ♂, Taghrart Ounakrim Cave, 04 Nov 2019, Moutaouakil S. leg., MHNM ZAI07 • 1 ♂, Tigmi N'Dou Akkal Cave, 25 Feb 2020, Moutaouakil S. leg., MHNM ZAI08.

Diagnosis. Telson with double-rounded tips. Antennula with eight petaliform aesthetascs. Antennal flagellum with six articles. Molar penicil of mandible with two plumose setae. Male pleopod 1 endopod with four wide scales at apex inner margin. Male pleopod 2 endopod thick and about 1.5× as long as exopod.

Description. Maximum body length: ♂ 5 mm, ♀ 6 mm. Body outline as in Fig. 2A. Colourless, dorsal surface tuberculate with tubercles arranged as follows: cephalon with four rows of tubercles; pereonite 1 with three rows of tubercles; pereonites 2–7 with two rows; pleonites 1 and 2 with one row of tubercles each, pleonites 3–5 and telson smooth, the latter about four times wider than long (Fig. 10B). Pleon narrower than pereon. Antennula with three articles (Fig. 8E), second article with one row of short setae on outer margin, third article with one row of eight petaliform aesthetascs along outer margin. Antenna long and slender (Fig. 8B), flagellum of six articles. Mandible with molar penicil consisting of two branches (Fig. 9C, D), basal one more than twice as long as the other; left mandible with 2+1 penicils, right with 1+1 penicils. Maxillula (Fig. 2B, C) outer endite with 12 simple teeth and one long seta, inner branch without setae, penicils or spines. Maxilla (Fig. 2D) rounded and covered with thin setae and one row of thick setae. Maxilliped (Fig. 2E) endite with long apical penicil; epipodite bearing fringe of thin setae on outer margin. Pereopods with long plumose dactylar seta (Fig. 3A–G). Uropods (Fig. 10B) with protopod slightly longer than wide; exopod bearing one row of scales on inner margin; endopod wider and longer than exopod.

Male. Pleopod 1 (Figs 4A, 10E) endopod with distal part pointed bent inwards, bearing four large transversal scales on the inner side; exopod triangular with no scale-setae (Fig. 4B). Pleopod 2 (Figs 4C, 10H) endopod thick and about 1/5 as long as exopod; exopod with one to two setae on apex (Fig. 4D).

Etymology. The name refers to the cave where the specimens were found first: Imi Ougoug Cave.
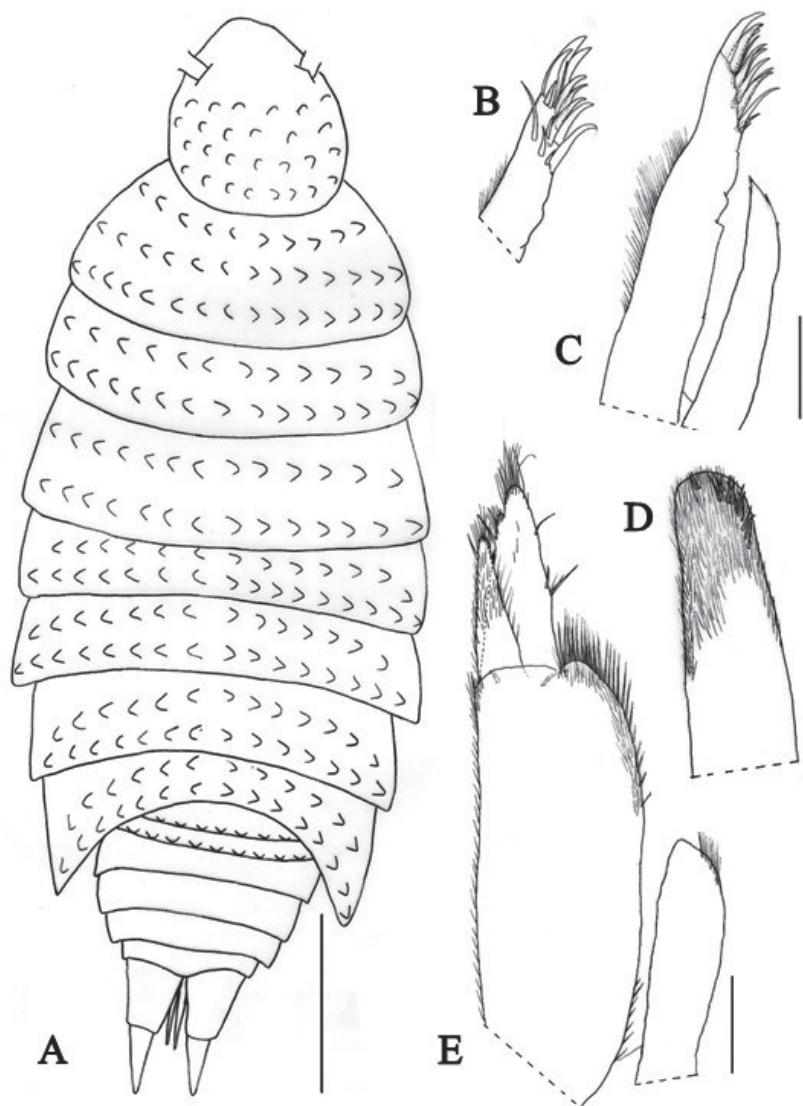
Figure 2. *Castellanethes ougougensis* sp. nov. ♂ specimen from Imi Ougoug Cave **A** adult specimen, dorsal view **B, C** maxillula **D** maxilla **E** maxilliped. Scale bars: 1 mm (**A**); 0.1 mm (**B–E**).

Castellanethes ighousi Moutaouakil & Boulanouar, sp. nov.
https://zoobank.org/B309B998-9962-411A-A97B-71E3497AD0C4
Figs 5–7, 8C, F, 9E, F, 10C, F, I, 11G–I, 12F

Material examined. *Holotype*: Morocco • 1 ♂ (mounted on a slide), Agadir-Imi Ougoug Cave, alt. 773 m, 22 May 2016, Moutaouakil S. leg., MHNM ZAI02. *Paratypes*: Morocco • 2 ♀, Imi Ougoug Cave, 29 Dec 2019, Moutaouakil S. leg., MHNM ZAI09 • 1 ♂, Tigmi N'Dou Akkal Cave, 25 Feb 2020, Moutaouakil S. leg., MHNM ZAI10.
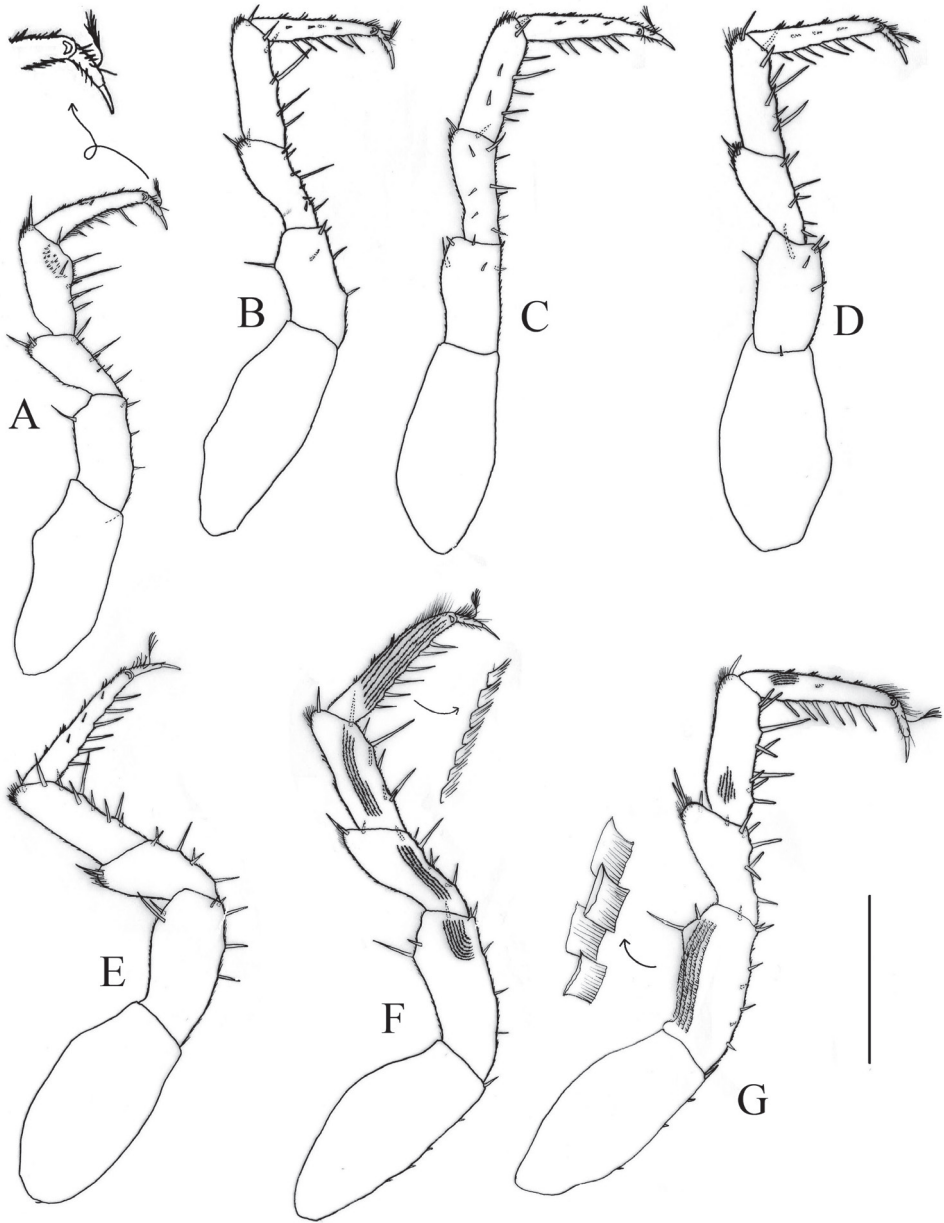
Figure 3. *Castellanethes ougougensis* sp. nov. ♂ specimen from Imi Ougoug Cave **A** pereopod 1 **B** pereopod 2 **C** pereopod 3 **D** pereopod 4 **E** pereopod 5 **F** pereopod 6 **G** pereopod 7. Scale bar: 0.5 mm.

Diagnosis. Telson with double-rounded tips. Antennula with three petaliform aesthetascs. Antennal flagellum with five articles. Molar penicil of mandible with two plumose setae. Male pleopod 1 endopod with four scales at the apex inner margin. Male pleopod 2 endopod stout with distal part tapering.
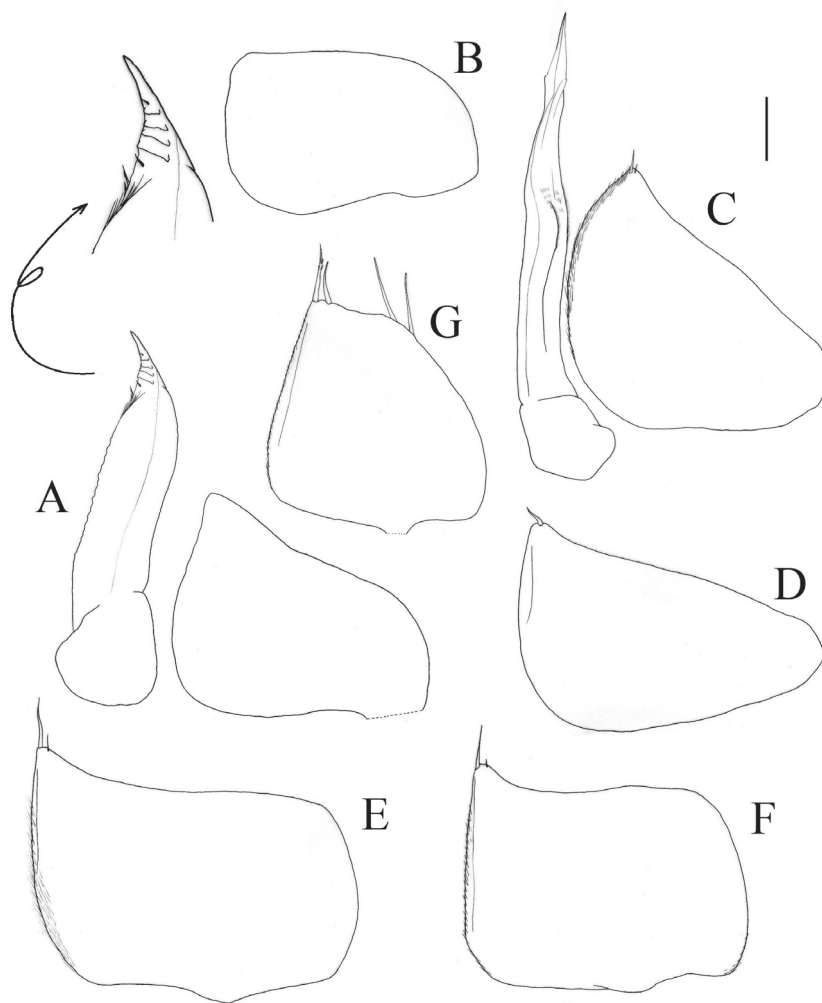
Figure 4. *Castellanethes ougougensis* sp. nov. from Imi Ougoug Cave **A** male pleopod 1 **B** female pleopod 1 exopod **C** male pleopod 2 **D** female pleopod 2 exopod **E** male pleopod 3 **F** male pleopod 4 **G** male pleopod 5. Scale bar: 0.1 mm.

Description. Maximum body length: ♂ and ♀ 8 mm. Body outline as in Fig. 5A. Colourless body, dorsal surface slightly granulated. Pleon narrower than pereon. Telson reduced, about three times wider than long (Fig. 10C). Antennula with three articles; third article with three petaliform aesthetascs (Fig. 8F). Antenna long; articles of peduncle with small-scale setae and simple setae, flagellum of five articles (Fig. 8C). Mandible with molar penicil consisting of two branches (Fig. 9E, F). Maxillula (Fig. 5B) outer endite with nine to ten simple teeth, two or three serrated setae and one long simple seta; inner endite with one row of small simple setae on inner distal margin. Maxilla (Fig. 5C) rounded and covered with many thin simple setae and a row of thick setae. Maxilliped (Fig. 5D) endite with long apical penicil; epipo-
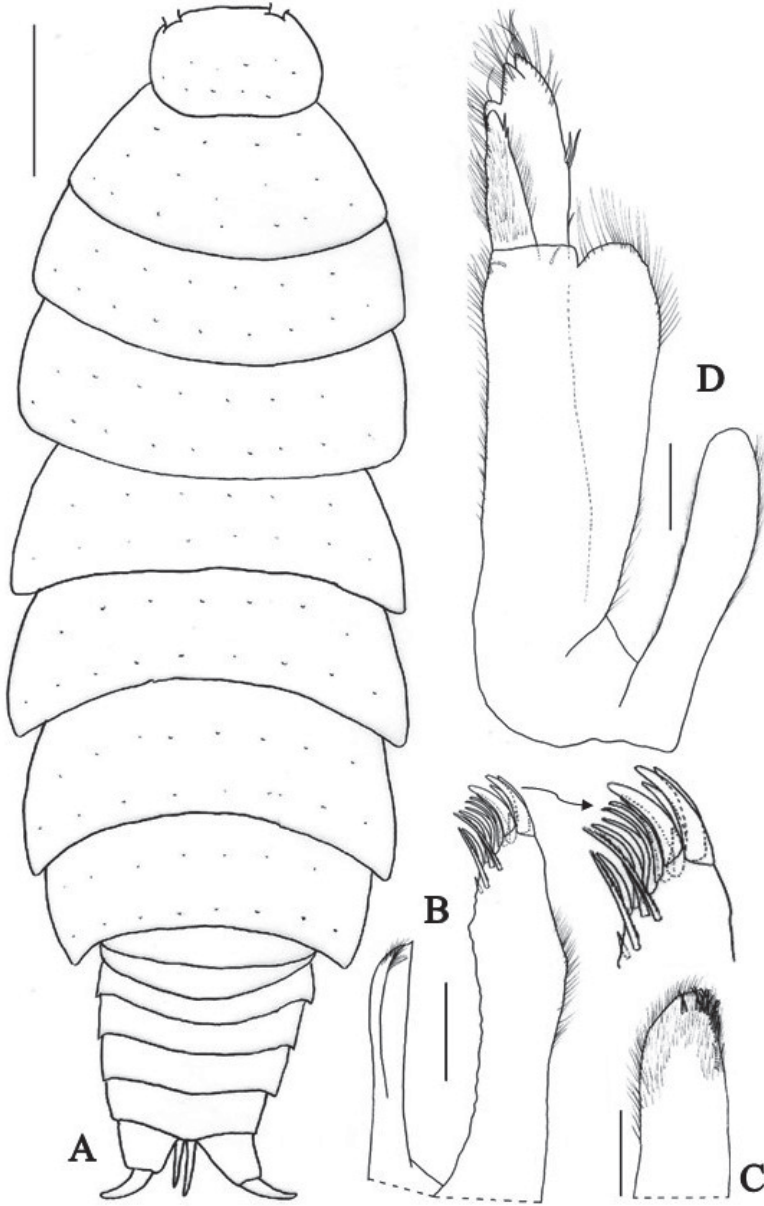
Figure 5. *Castellanethes ighousi* sp. nov. ♂ specimen from Imi Ougoug Cave **A** adult specimen, dorsal view **B** maxillula **C** maxilla **D** maxilliped. Scale bars: 1 mm (**A**); 0.1 mm (**B–D**).

dite hairy with a row of elongate setae on distal margin. Dactylar seta bifurcated and plumose. Pereopods increase in size, with pereopod 7 about twice as long as pereopod 1 (Fig. 6A–G). Uropods (Fig. 10C) with protopod wider than long; exopod as thick as endopod, bearing one row of scales on inner margin; endopod bearing many short setae on apex and one long seta.
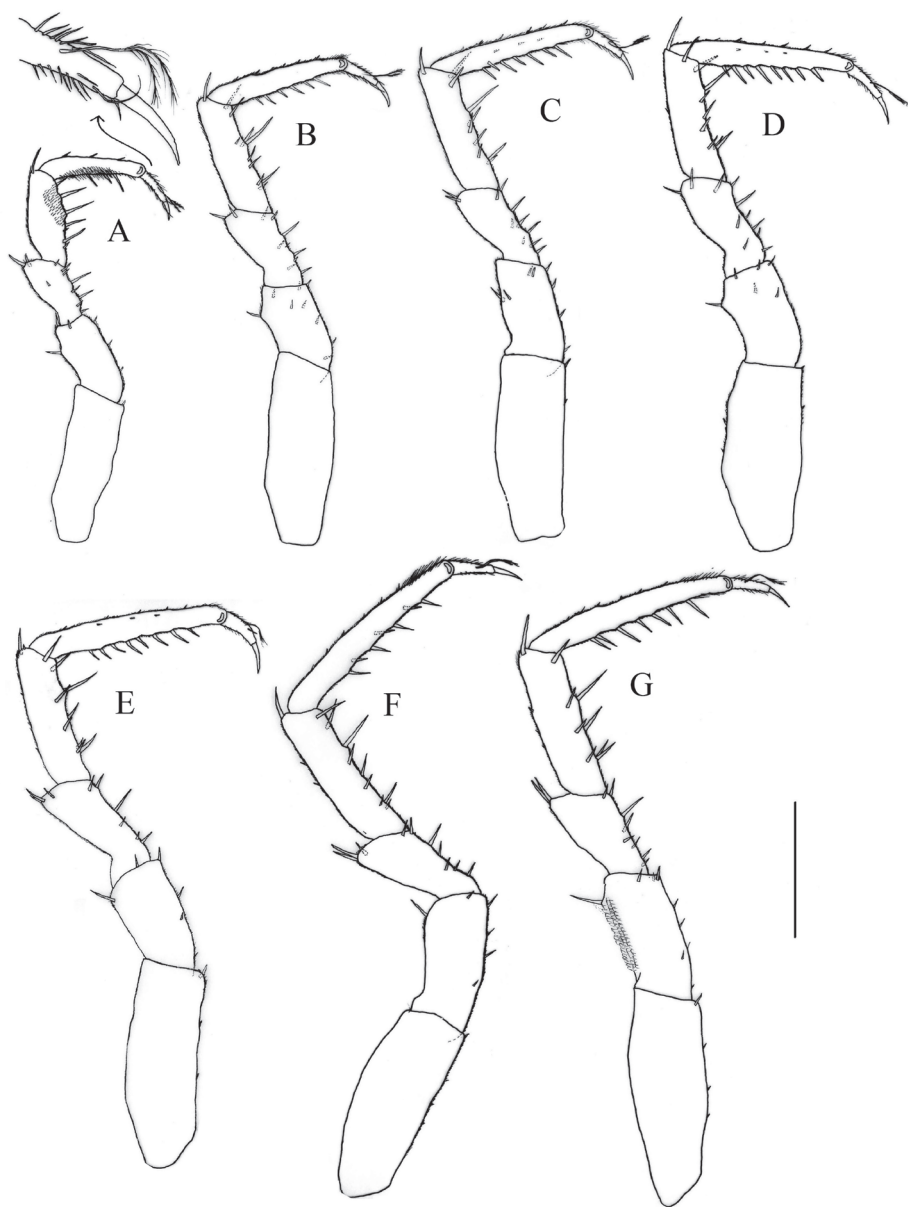
Figure 6. *Castellanethes ighousi* sp. nov. ♂ specimen from Imi Ougoug Cave **A** pereopod 1 **B** pereopod 2 **C** pereopod 3 **D** pereopod 4 **E** pereopod 5 **F** pereopod 6 **G** pereopod 7. Scale bar: 0.5 mm.

Male. Pleopod 1 (Figs 7A, 10F) endopod with pointed tip bent inwards, bearing four scales on inner side; exopod triangular bearing one seta at apex and thin simple setae on inner margin (Fig. 7B). Pleopod 2 (Figs 7C, 10I) endopod bent outwards
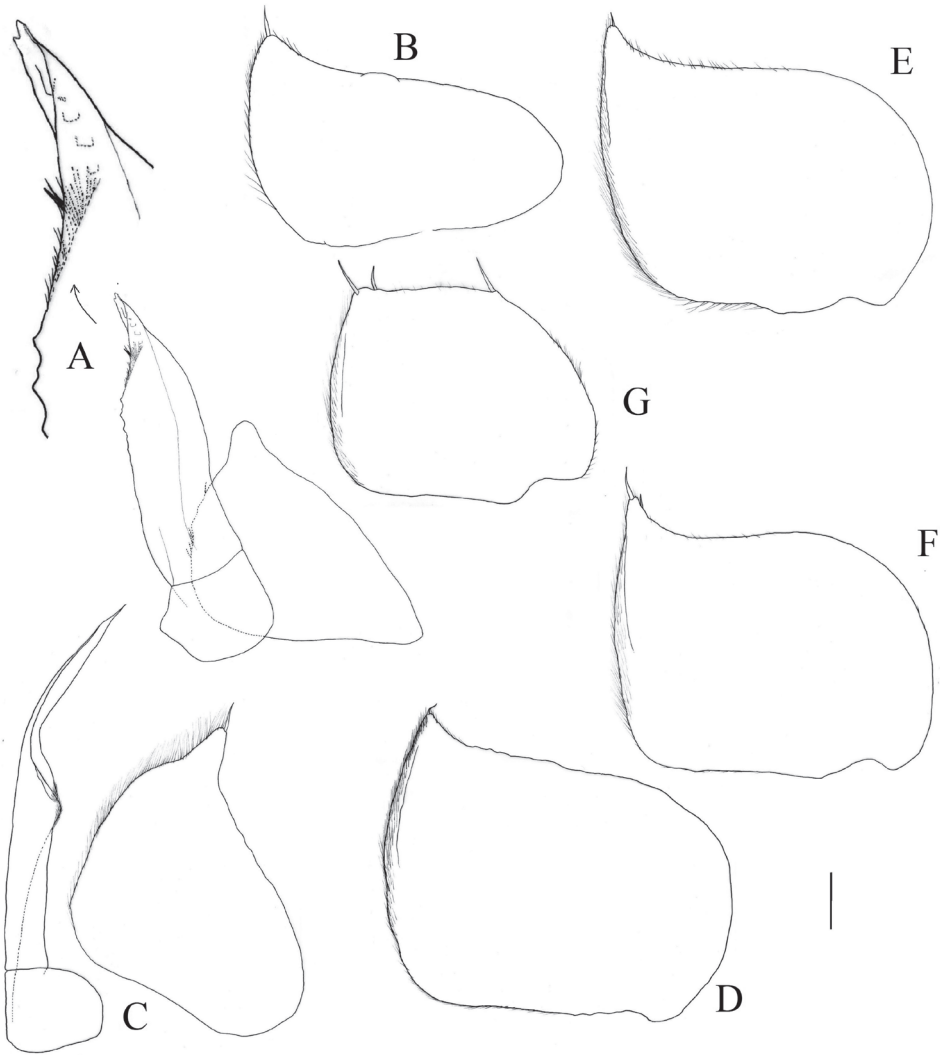
Figure 7. *Castellanethes ighousi* sp. nov. from Imi Ougoug Cave **A** male pleopod 1 **B** female pleopod 1 exopod **C** male pleopod 2 **D** female pleopod 2 exopod **E** male pleopod 3 **F** male pleopod 4 **G** male pleopod 5. Scale bar: 0.1 mm.

with wide proximal half and thin distal part; exopod with small simple setae on inner margin (Fig. 7D).

Etymology. The new species is named after Mr. Ighous Abdelaziz, the speleologist with whom one of the authors (SM) visited Imi Ougoug Cave the first time and who passed away 5 years ago. He was one of the pioneers of speleology in the Agadir region and one of the founders of the Association Sportive de Spéléologie (ASS).
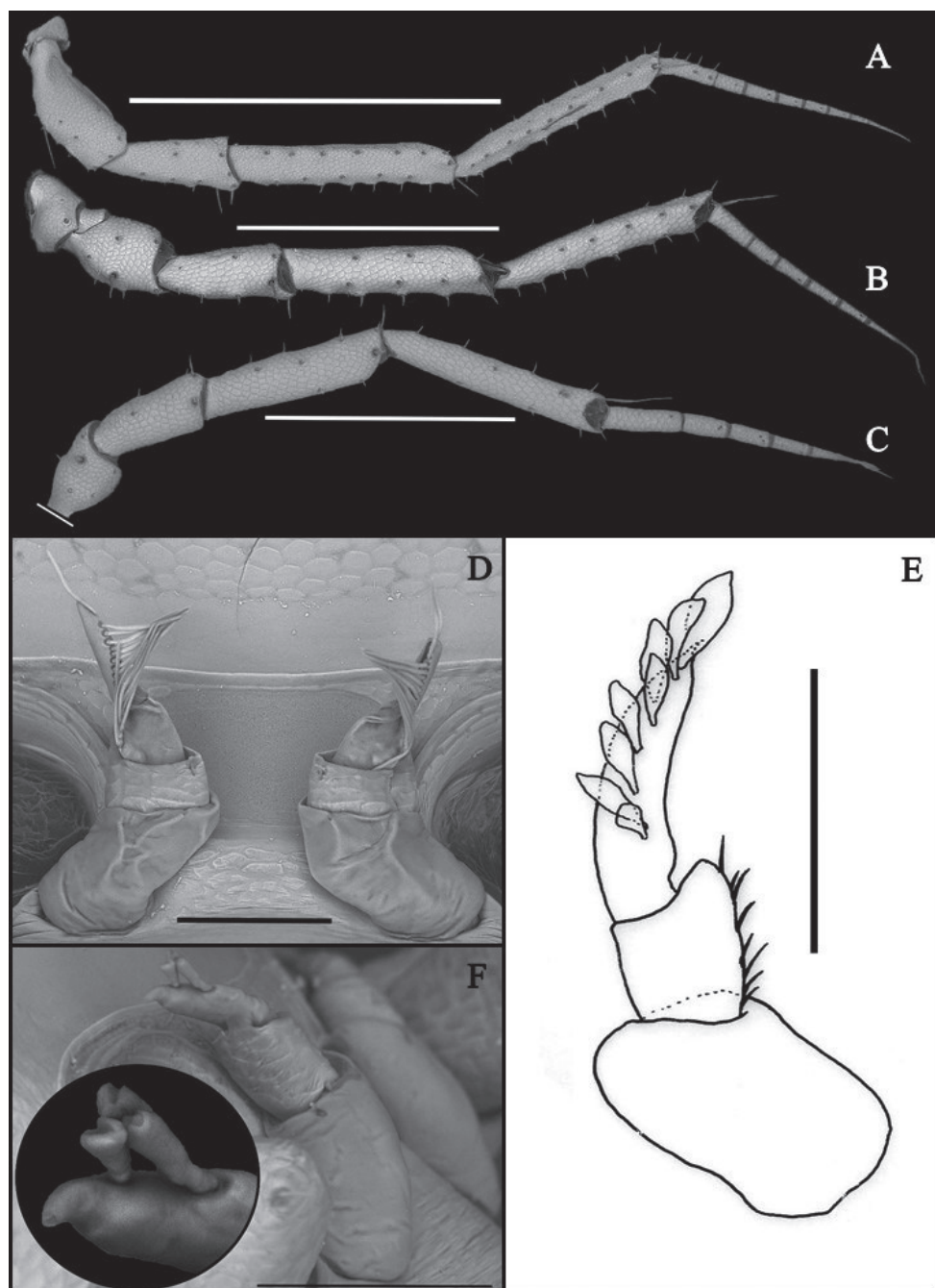
Figure 8. *Castellanethes soloisensis* from Goran Cave **A** antenna **D** antennula. *Castellanethes ougougensis* sp. nov. from Imi Ougoug Cave **B** antenna **E** antennula. *Castellanethes ighousi* sp. nov. from Imi Ougoug Cave **C** antenna **F** antennula. Scale bars: 1 mm (**A**); 500 μm (**B, C**); 100 μm (**D–F**).
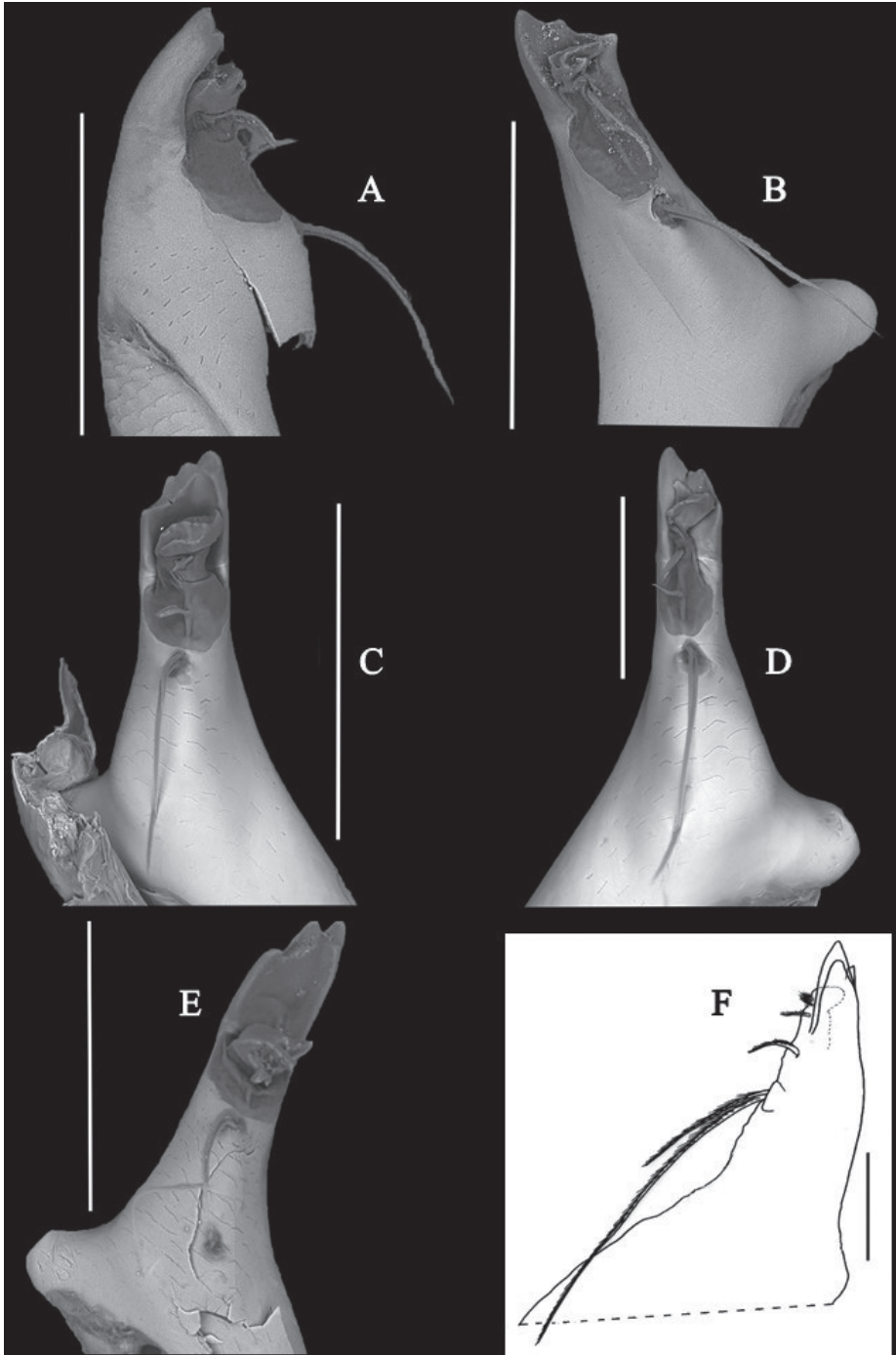
Figure 9. *Castellanethes soloisensis* from Goran Cave **A** left mandible **B** right mandible. *Castellanethes ougougensis* sp. nov. from Imi Ougoug Cave **C** left mandible **D** right mandible. *Castellanethes ighousi* sp. nov. from Imi Ougoug Cave **E** left mandible **F** right mandible. Scale bar: 200 μm (**A–F**).
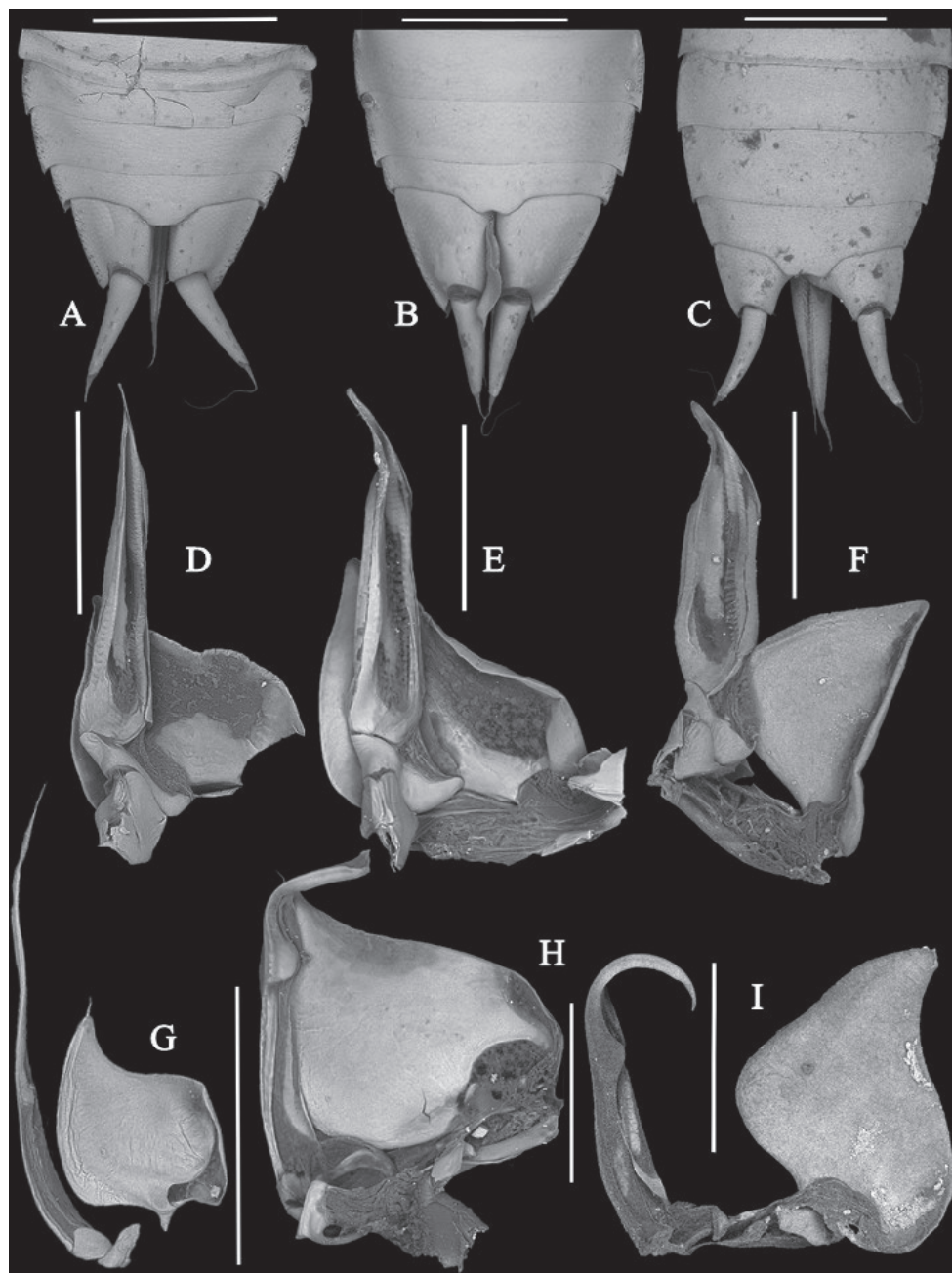
Figure 10. *Castellanethes soloisensis* from Goran Cave **A** telson + uropods **D** male pleopod 1 **G** male pleopod 2. *Castellanethes ougougensis* sp. nov. from Imi Ougoug Cave **B** telson + uropods **E** male pleopod 1 **H** male pleopod 2. *Castellanethes ighousi* sp. nov. from Imi Ougoug Cave **C** telson + uropods **F** male pleopod 1 **I** male pleopod 2. Scale bar: 500 µm (**A, C, G**); 400 µm (**B**); 300 µm (**D, F, I**); 200 µm (**E, H**).
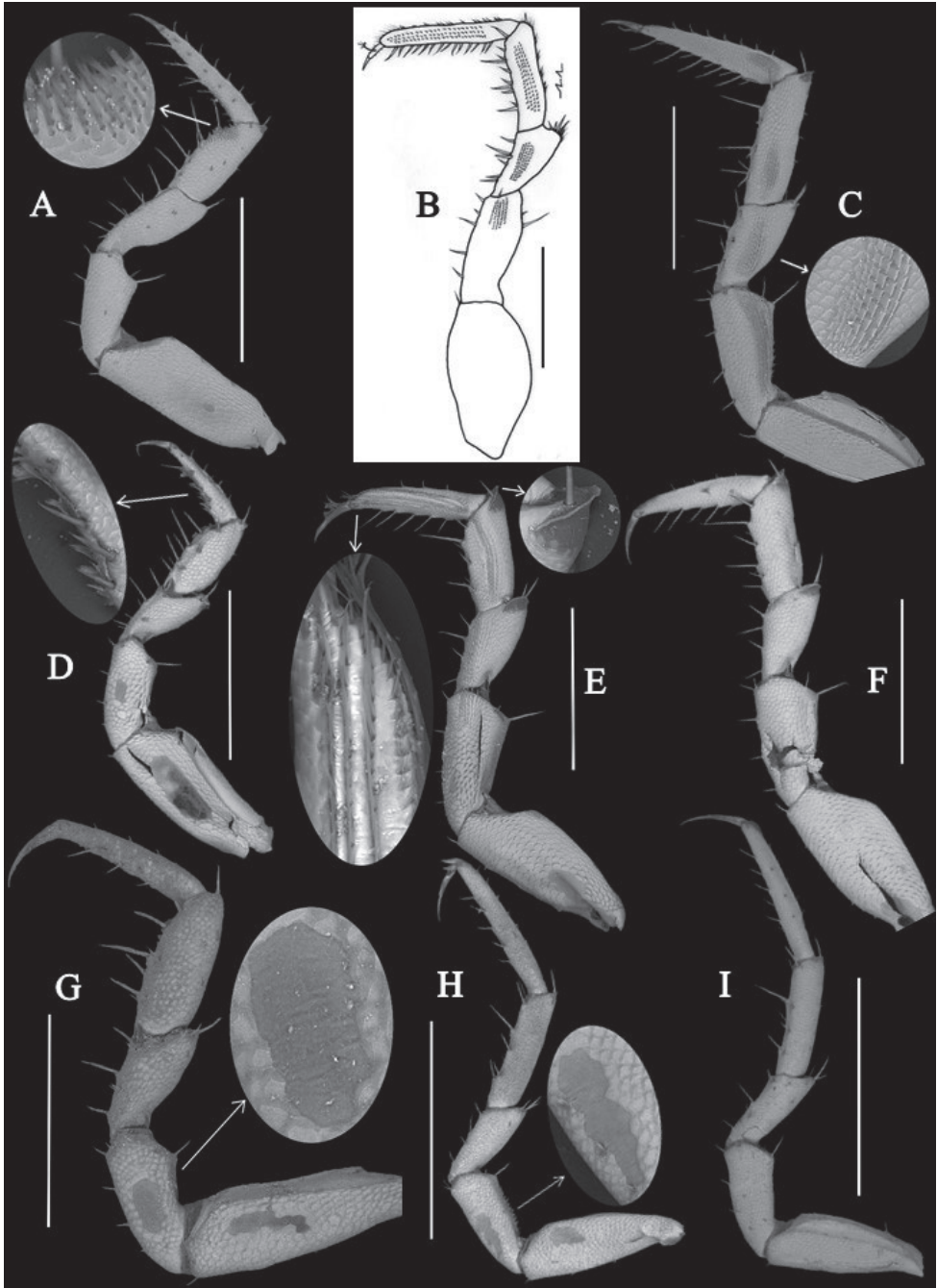
Figure 11. *Castellanethes soloisensis* ♂ specimen from Goran Cave **A** pereopod 1 **B** pereopod 6 **C** pereopod 7. *Castellanethes ougougensis* sp. nov. ♂ specimen from Imi Ougoug Cave **D** pereopod 1 **E** pereopod 6 **F** pereopod 7. *Castellanethes ighousi* sp. nov. ♂ specimen from Imi Ougoug Cave **G** pereopod 1 **H** pereopod 6 **I** pereopod 7. Scale bar: 1 mm (**H, I**); 500 μm (**A–G**).

Figure 12. Presentation of the type locality (Imi Ougoug Cave) **A** entrance of the Imi Ougoug Cave **B** Imi Ougoug Cave topography **C** external area at Talmat River **D** aquatic habitat inside Imi Ougoug Cave **E** terrestrial habitat **F** *Castellanethes ighousi* in a pond **G** Scolopendromorpha/isopod's predator **H** *Castellanethes ougougensis.*

Discussion

Castellanethes ougougensis sp. nov. and *C. ighousi* sp. nov. were placed into the genus *Castellanethes* because they possess 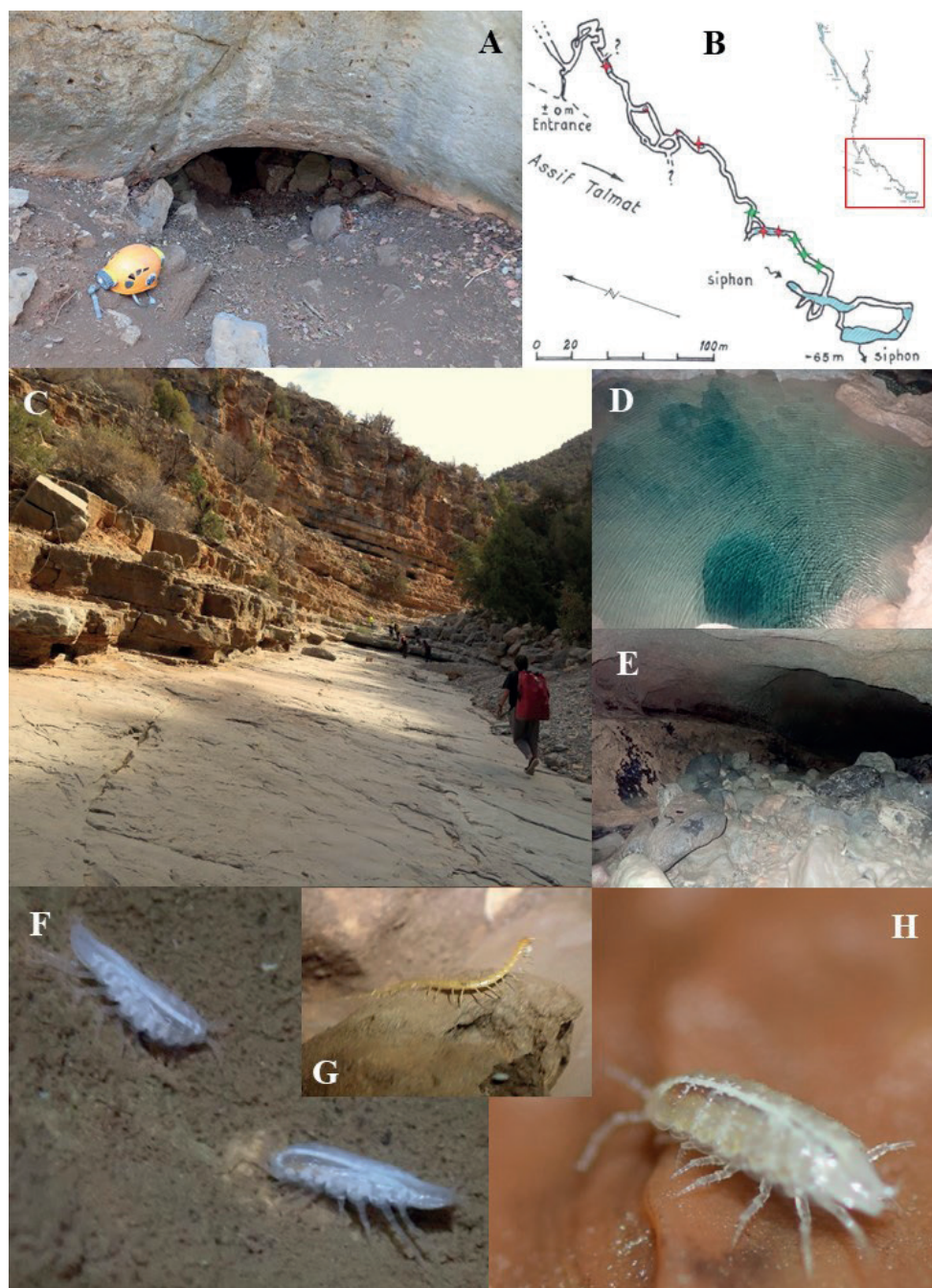all the characters defined by Brian (1952) and Taiti and Gardini (2022). They are described as a new species since they are distinguishable from the other five species of the genus.

Castellanethes ougougensis sp. nov. differs from *Castellanethes ighousi* sp. nov. by remarkable tubercles (Fig. 2A) instead of reduced ones on the dorsal surface (Fig. 5A); antennula with one row of eight petaliform aesthetascs on the third article (Fig. 8E) instead of three petaliform aesthetascs (Fig. 8F); antennal flagellum with six articles instead of five (Fig. 8B, C); pereopods 1–7 subequal in length (Figs 3, 11D–F) instead of increasing in size with pereopod 7 about twice as long as pereopod 1 (Figs 6, 11G–I); male pleopods 1 endopod long and thin with pointed tip (Figs 4A, 10E) compared to *C. ighousi* sp. nov. pleopod 1 endopod which is wider and shorter (Figs 7A, 10F); male pleopod 2 endopod styliform and thick (Figs 4C, 10H) instead of having wide proximal half and thin distal part (Figs 7C, 10I); uropods (Fig. 10B) with protopod longer than wide and thin exopod bearing two to three setae on the apex and a row of scales on inner margin, while *C. ighousi* sp. nov. uropod protopod is wider than long and exopod as thick as endopod (Fig. 10C).

The two new species differ from the five known species of this genus by remarkable characters. *Castellanethes soloisensis* is characterised by an antennula with spiniform aesthetascs (Fig. 8D), antennal flagellum with eight articles (Fig. 8A), male pleopod 1 endopod with nine scales at the apex inner margin (personal observation) and male pleopod 2 endopod styliform and more than twice as long as exopod (Fig. 10G). *Castellanethes velox* has a mandible with molar penicil consisting of one plumose seta, a telson with triangular distal part and antenna with four flagellar articles. *Castellanethes insularis* shows presence of eyes and mandible with molar penicil consisting of four setae. *Castellanethes fluviatilis* possesses antennula with only two thickset aesthetascs. Finally, *Castellanethes sanfilippoi* is distinguished by the antennal flagellum with four articles and pleopod 2 endopod longer and thickset.

Habitat

Imi Ougoug is a limestone cave, also known as "Ifri Ouado" which means "the blowing cave" in Amazigh, a name that was given by local people due to the air current that comes out of it. It is also known as "Grotte des Araignées", which means "spider cave" in French, referring to the large number of harvestmen found inside. This cave is located 43 km northeast of Agadir City, at the bottom of a cliff overlooking the Talmat River. It has a small entrance with a narrow zig-zag passage that splits after reaching 10 m from the entrance and leads on the right to the southern passage of the Imi Ougoug Cave. This passage, which consists of a sinuous path with alternating dry areas and ponds, ends 435 m from the entrance on a siphoning lake (Fig. 12B) (MET 1981).

The two Isopoda species were observed on this strand; *Castellanethes ougougensis* sp. nov. (Fig. 12H) was mainly observed in terrestrial environments, while *Castellanethes ighousi* sp. nov. (Fig. 12F) seems to be aquatic. Their co-occurrence in this cave can be explained by the different habitats they occupy, which contributes to a low niche overlap and reduced (if any existing) interspecific competition (Taiti et al. 2018).

This part of the cave, inhabited by the two species, is characterised by a temperature of 20.8 °C and a humidity reaching 91.2% compared to the entrance zone which is drier and colder (humidity = 73% and temperature = 17.1 °C). The average water temperature is 22.5 °C, dissolved oxygen is 4.51 mg/l (60.9%), pH is 7.04 and conductivity is 360 µS/cm.

As this cave was subject to flooding in the past by the Talmat River, it received a lot of debris from the outside. However, since this river has been dry for a number of years, the main source of organic matter for the isopods is probably wood fragments observed in some areas, bat guano and microbial communities. This cave is connected with the "Chauves-souris" Cave which hosts a large colony of bats.

Notes on conservation

Several other troglobitic and troglomorphic species inhabit the Imi Ougoug Cave: the liocranid spider *Agraecina agadirensis*, the staphylinid beetle *Apteranillus bichaini* and the cirolanid isopod *Typhlocirolana haouzensis* Boutin, Boulanouar, Coineau & Messouli, 2002 (personal observation), the latter has been recorded in many regions in Morocco (in the south-eastern regions of the High Atlas, in the western area and in further northern regions (the Peri-Rifian area)), but never from caves in upstream areas of the Atlas Mountains (Boutin et al. 2002). However, this stygobitic species has been observed in the left passage of Imi Ougoug Cave at an elevation of 800 m.

Other troglobitic species not yet described have also been observed: a japygid (Diplura), a centipede (Scolopendromorpha), a dysderid spider and many paradoxosomatid specimens. Some of these species are potential predators of the isopods (Pagés 1951; Arnedo et al. 2007).

The small distributional range of these troglobitic species, their physiological specialisation and the simplified ecological communities to which they belong, imply a greater vulnerability of these species and a higher risk of trophic web disruption. Considering their inability to modify their distribution in response to eventual habitat disturbances, such species may be considered threatened, especially when taking into account alterations arising from climate change (Mammola and Isaia 2017). In addition, this Cave has become the main destination for local associations and tourist groups (Lecigne and Moutaouakil 2021). Moreover and given the cave morphology, the different habitats cannot be avoided while progressing inside. Therefore, the protection of this cave ecosystem through legislative acts is highly recommended.

The lack of studies on troglobitic isopods in Morocco leads to a deficiency regarding the actual biodiversity and distribution of this group. Therefore, quantifing

the threats that endanger their existence is not currently an easy task. This study is a contribution to the knowledge of this particular group and an enhancement of the knowledge of cave-restricted invertebrate fauna in Morocco.

Acknowledgements

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RESEARCH ARTICLE

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Protocol for lens removal in embryonic fish and its application on the developmental effects of eye regression

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Abstract

The lens plays a central role in the development of the optic cup. In fish, regression of the eye early in development affects the development of the craniofacial skeleton, the size of the olfactory pits, the optic nerve, and the tectum. Lens removal further affects olfaction, prey capture, and aggression. The similarity of the fish eye to other vertebrates is the basis for its use as an excellent animal model of human defects. Questions regarding the effects of eye regression are specifically well-suited to be addressed by using fish from the genus *Astyanax*. The species has two morphs; an eyeless cave morph and an eyed, surface morph. In the cavefish, a lens initially develops in embryos, but then degenerates by apoptosis. The cavefish retina is subsequently disorganized, degenerates, and retinal growth is arrested. The same effect is observed in surface fish when the lens is removed or exchanged for a cavefish lens. While studies can greatly benefit from a control group of surface fish with regressed eyes brought through lensectomies, few studies include them because of technical difficulties and the low survivorship of embryos that undergo this procedure. Here we describe a technique with significant modification for improvement for conducting lensectomy in one-day-old *Astyanax* and other fish, including zebrafish. Yields of up to 30 live embryos were obtained using this technique from a single spawn, thus enabling studies that require large sample sizes.

Keywords

Introduction

The lens plays an important role in the development of the optic cup (Thut et al. 2001; Yamamoto and Jeffery 2001). Using fish from the genus *Astyanax*, questions regarding the effects of eye regression can be addressed. The species has two morphs; an eyeless cave morph and an eyed, surface morph that remain inter-fertile. The blind Mexican tetra is a model system in evolutionary developmental biology which has provided an unprecedented understanding of the genetic and developmental controls of features associated with being eyeless and living in the continuous darkness of the caves (Jeffery 2001). *Astyanax* are well fitted for laboratory research and suitable for experimental manipulation due to several advantageous characteristics, including small size, high fecundity, short lifecycle, and relative ease of care. Since the genome of both surface and cave morphs has been sequenced, abundant genetic and molecular resources are available to support research in *Astyanax* (McGaugh et al. 2014; Imarazene et al. 2021; Warren et al. 2021). In addition, the similarity of the fish eye to those of other vertebrates provides the basis for its use as an excellent animal model of human eye defects (Schmitt et al. 1994; Malicki 2000; Malicki et al. 2002; Avanesov and Malicki 2004; Cavodeassi and Wilson 2019).

The role of the lens in eye development has been studied in *Astyanax*. Lens development occurs rapidly in this species. By 18.5 hours post-fertilization (hpf), the *Astyanax* lens has rounded from the placode and is visible (Hinaux et al. 2011; Devos et al. 2021). A lens and a layered retina initially develop in cavefish embryos, but the lens rapidly undergoes massive apoptosis after one day of development (Yamamoto and Jeffery 2000). The cavefish retina is subsequently disorganized, apoptotic cells appear, the photoreceptor layer degenerates, and retinal growth is arrested (Alunni et al. 2007; Strickler et al. 2007). When a surface fish lens is transplanted into a cavefish's developing optic cup, it stimulates growth and development, restoring optic tissues lost during cavefish evolution. Conversely, eye growth and development are retarded following transplantation of a cavefish lens into a surface fish optic cup or through a lensectomy early in development (Yamamoto and Jeffery 2000). These results show that evolutionary changes in signal(s) from the lens are involved in cavefish eye degeneration.

Manipulations of eye formation by transplantation of the embryonic lens or by lensectomy have been crucial to understanding eye-dependent and eye-independent processes. Cavefish craniofacial skeletons and the size of the olfactory pits in adults were found to correlate with eye development (Yamamoto et al. 2003). Likewise, the lens indirectly influences the optic nerve and tectum development in blind cavefish (Soares et al. 2004). Lensectomy studies can also help inform the effect of developing eyes on behavior. For example, surface fish raised in the dark or after embryonic lens ablation leading to eye degeneration have improved olfactory detection capacities (Blin et al. 2018). However, when analyzing prey capture competition assays on these fish, the eye-dependent developmental processes were found not to be the main determinant for enhanced prey capture skills (Espinasa et al. 2014). Aggression is another example. Surface-dwelling individuals are highly aggressive, whereas their blind, cave-dwelling counterparts tend to show little or no aggressive behavior. Surface fish blinded early in their embryonic development through lensectomy remain highly aggressive as

adults (Espinasa et al. 2015). Thus, aggression in *Astyanax* can be triggered without visual stimuli in surface fish.

In this paper we describe the technical approach for removing a lens from a developing *Astyanax* surface morph that is generalizable to other species. Although this technique has been used in the past, prior studies reported low survivorship and a substantial degree of difficulty. The modified protocol described herein allows for much faster and more reliable removal of lenses that can yield high survivorship. We aim to provide a clear roadmap for other interested researchers to perform this experimental technique.

Protocol

Animals were treated according to the French and European regulations for the use of animals in research. SR's authorization for using animals in research, including *Astyanax mexicanus*, is 91–116. The Paris-Saclay Institute's animal facility authorization number is B91-272-108. Specimens are those used in Espinasa et al. (2014) and Blin et al. (2018).

Part 1: Preparing the embryos

Astyanax breeding has been described elsewhere (Elipot et al. 2014; Peuß et al. 2019; Ma et al. 2021). Briefly, an increase of temperature from 22 °C to 26.5 °C in the first three days of a breeding week provides two-to-three consecutive spawning days with maximal numbers of high-quality embryos, which is then followed by a decrease of temperature from 26.5 °C to 22 °C during the last three days of the spawning week. Most spawning occurs at night for both surface and cavefish. In the morning, collect embryos in 100 × 15 mm Petri dishes, sort and clean them, and transfer them into embryo medium (EM-Westerfield 2000) with methylene blue. An alternative is to conduct in-vitro fertilization.

Keep the embryos in EM in a 23 °C incubator until the desired stage. The lens becomes visible in *Astyanax* kept at 23 °C at 18.5 hpf. Hatching occurs at 24.5–28 hpf. The lens enters apoptosis at about 25 hpf. When conducting lensectomy on surface fish to replicate the effects of lens degeneration in cavefish, the optimum time is 1–3 dpf, or within 48 hrs after hatching. If lensectomy is to be conducted before hatching, remove the chorion manually with two pairs of sharp forceps, and incubate the embryos in 0.2% EDTA in Calcium-free Zebrafish Ringer's (ZFR) for 30 minutes.

Part 2: Preparing dissection needles

In previous protocols, two needles were used. One with a blunt tip needle made of a thin tungsten wire and a second one with a sharp tip made by holding the tungsten wire over a Bunsen burner for 1–1.5 minutes, burning off the metal, and creating a very fine tip). Previous protocols instructed lensectomies to be conducted by hand under a microscope. Since the lens is only about 50 μm, extreme precision is required to

ablate the lens without harming other structures. Normal tremor of the hands makes this extremely challenging, even for highly trained people.

In this improved protocol, instead of using tungsten needles held by hand, microinjection needles were made from glass capillaries mainly with a Narishige's PC-10 Dual-Stage Glass Micropipette Puller, with the puller was set to a one step weighted pull at 70.5 °C. Other brand micropipette pullers were tested and found to give similar results. Borosilicate glass capillaries are heated and pulled to get extremely fine and sharp needles, similar to those used for cell injections (Fig. 1A, B). These needles are then attached to a manual micromanipulator (Type MM33 Rechts; Märzhäuser, Wetzlar, Germany). The micromanipulator allows for precise movements in the X, Y, and Z directions, eliminating hand tremor effects and maximizing the precision of movements. We found that the best technique was with one hand controlling the micromanipulator, while the other hand moved the petri dish that holds the specimen (Fig. 1C). This combination allowed for the best combination of dexterity and efficiency of movements.
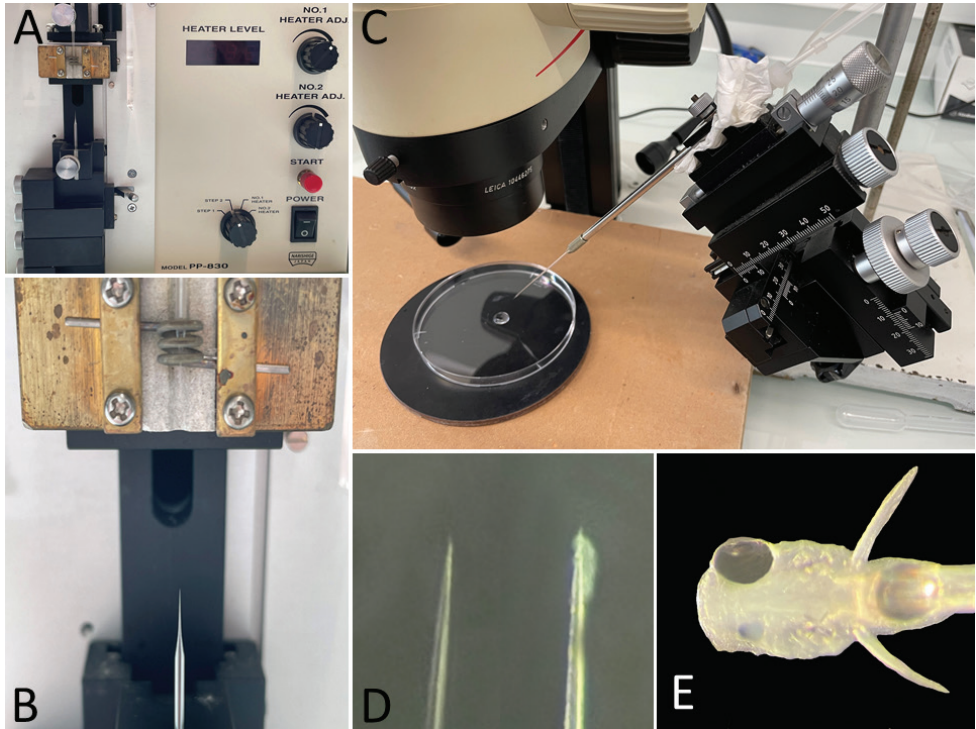
Figure 1. A for the preparation of dissection needles, microinjection needles are made from borosilicate glass capillaries with a Micropipette Puller **B** glass capillaries are heated and pulled to get extremely fine and sharp needles **C** instead of manipulating the dissection needle by hand, a micromanipulator is used. This dramatically reduces jittery movements that can puncture neighboring structures such as the brain or the heart. The micromanipulator allows precise puncturing around the lens with the needle's movements controlled easily at less than 5 μm **D** clean needles are essential. Throughout the procedure, the needle progressively gets covered with a fatty substance that essentially blunts the needle. It is best to exchange it for a new one **E** embryo a week after a one-sided lensectomy in dorsal view. The left, lensectomized eye is regressing.

Part 3: Preparing reagents and equipment

-   Embryo medium (EM; pH 7.0, per liter, contains: 10 ml Hanks Solution #1, 1 ml Hanks Solution #2, 10ml Hanks Solution #4, 10 ml Hanks Solution #5, 0.35 g sodium bicarbonate, 300 uL of 2M HCl, penicillin-streptomycin 500,000U) As in The Zebrafish Book (University of Oregon Press, 2000).
-   Anesthetizing solution:10 ml EM, 1 mg ms-222 (tricaine methanesulfonate), 1 mg $NaHCO_3$.
-   2% low melting agarose: 10 ml EM, 1 mg ms-222 (tricaine methanesulfonate), 1 mg $NaHCO_3$, 0.2 gr low melting agarose.
-   Pulled glass capillaries.
-   Dissection needle with a thin tungsten wire blunt tip.
-   Mini scalpel.
-   Plastic pipette dropper (wide mouth so the embryo can easily fit).
-   200 μl pipette.
-   Petri dishes.
-   Dissection microscope.
-   Manual micromanipulator.

Part 4: Lensectomy procedure

•   At the desired stage (40 hpf, for example), take the embryos kept in EM in the incubator. With a plastic pipette dropper, carefully transfer the embryos to the anesthetizing solution and incubate for about 30 seconds or until the embryos stop moving. The number of embryos depends on proficiency. We operated up to ten specimens simultaneously, but for best survival and efficiency, about three at a time is recommended.

•   With the pipette dropper, transfer anesthetized specimens to a petri dish (Fig. 2A). Before adding the agarose, absorb the excess liquid (Fig. 2B). It was found that, otherwise, the agarose surrounding the embryo would be diluted and would not grasp as firmly during the micromanipulations. However, caution should be used that the embryo does not dry before adding the agarose.

•   Add the 2% low melting agarose when the melted agarose is close to room temperature but before it solidifies on and around the embryo (Fig. 2C). Most embryos will naturally lie on their sides in the agarose. Reorient those that do not lie in this position so that one eye is facing up before the agarose solidifies. The depth at which the embryo lies within the agarose drastically changes the performance. When very deep, it is difficult to see the structures, and when traversing the agar, the needle gets deflected from its target. When barely covered, it risks detaching the specimen from the agar. We found that adding about 100 μl agarose worked well, but sometimes excess agarose must be reabsorbed when the specimen sinks or is attached to the petri dish.

•   Wait for the 2% agarose to solidify. For time efficiency, the Petri dish containing the samples can be put in a refrigerator for 1–2 min. It was also found convenient to use this time to make a pair of microinjection needles. After the agar solidifies, the Petri dish is transferred under a dissecting microscope (Fig. 1C). Microscopes of high quality,

good resolution, and good illumination are recommended for the best results. By its side, there will be the manual micromanipulator with the microinjection needle attached to it (Fig. 1C). Adding a few drops of liquid anesthetizing solution on top of the agarose is optional. Depending on the specific sample, it can enhance or reduce visual clarity.

• Using the microinjection needle, very carefully cut the lens out from the embryos using small strokes. Make sure to cut just close enough to the lens that it neither tears it, nor removes too much tissue from the rest of the eye. We developed three styles, each with its advantages and disadvantages:

Style 1: With the micromanipulator, slowly bring down the needle by the side of the lens. Pressure down until it makes a puncture in the surface ectoderm/cornea at the junction between the lens and optic cup (Fig. 3A). Depending on the specimen and how solid the
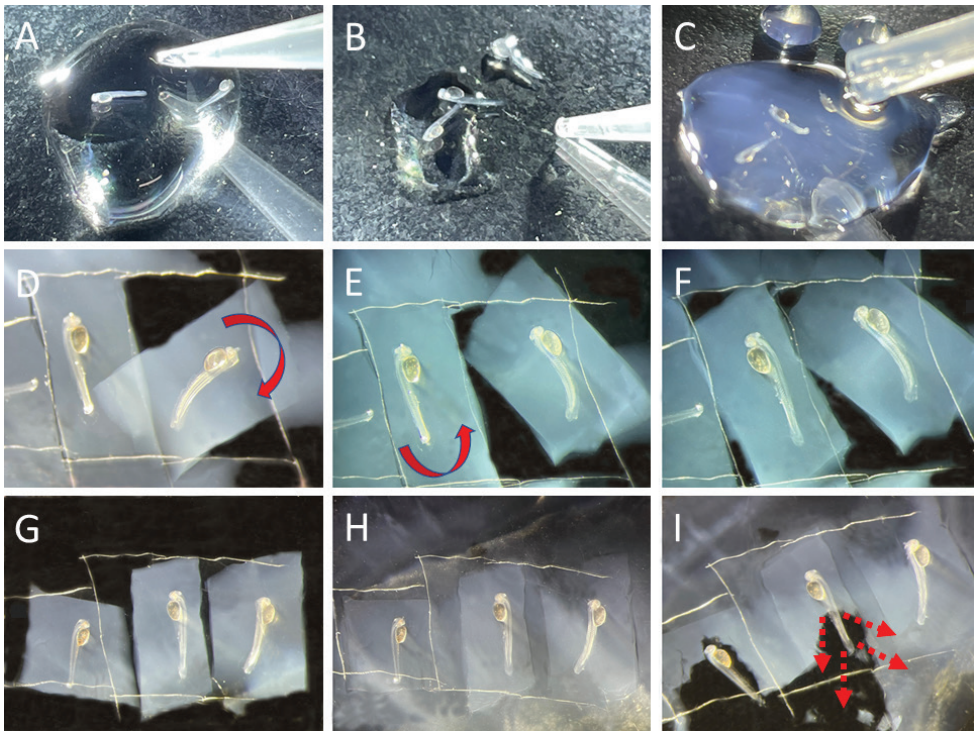
Figure 2. A specimens are transferred to a Petri dish after being in anesthetizing solution for 30 seconds or until embryos stop moving **B** absorb the excess liquid **C** add 2% agarose EM. The depth at which the embryo lies should not be too deep because the needle gets deflected from the target, and it is difficult to see the structures. However, it risks detaching the specimen from the agar when barely covered. After agarose solidifies, proceed with lensectomy **D–G** if lensectomy is to be done on both sides, with a scalpel, cut a rectangle of the agar around the specimen. Very gently slide the scalpel under the rectangle of agar with the specimen. Helped with twicers, flip around the agar slab, so the specimen is on the other side. More than one specimen can be done at a time to increase yield **H** add 2% agarose around the rectangle of agar or over the specimen if it was dislodged **I** after the second lensectomy is done, submerge in embryo media and gently dislodge the embryo from the agar with downward strokes starting around the tail and ending on the head.

agarose grabs the specimen, the eye can deform and sink slightly down with the pressure until the needle ruptures the tissue. Be careful that the needle does not go further down and punctures other structures, such as the brain or the heart. The use of the micromanipulator is a great improvement to the previous protocols that used a hand-held needle in this regard. After a hole is made with the micromanipulator, pull the needle out. With the hand holding the petri dish, slightly reposition the sample so the needle can make a different puncture at another site near the lens (Fig. 3B). Repeat these punctures with a circling pattern around the lens. Ten to fifteen punctures are performed (Fig. 3C). Afterward, with a coordinated motion of the hand holding the petri dish and the micromanipulator, insert the needle between punctures and gently pull the needle out to tear the tissue between punctures. Repeat this progressive tearing of tissue around the lens. Finally, put the needle on one side, under the lens, and push it out of the optic cup

Figure 3. Style #1 for doing lens ablations **A** with the micromanipulator, slowly bring down the needle by the side of the lens and pressure down until it makes a perforation on the surface ectoderm/cornea **B** repeat these punctures around the lens **C** insert the needle between punctures, and with a coordinated motion of the hand holding the petri dish and the micromanipulator, gently pull the needle out to tear the tissue between punctures **D** put the needle on one side, under the lens and push it out of the optic cup.
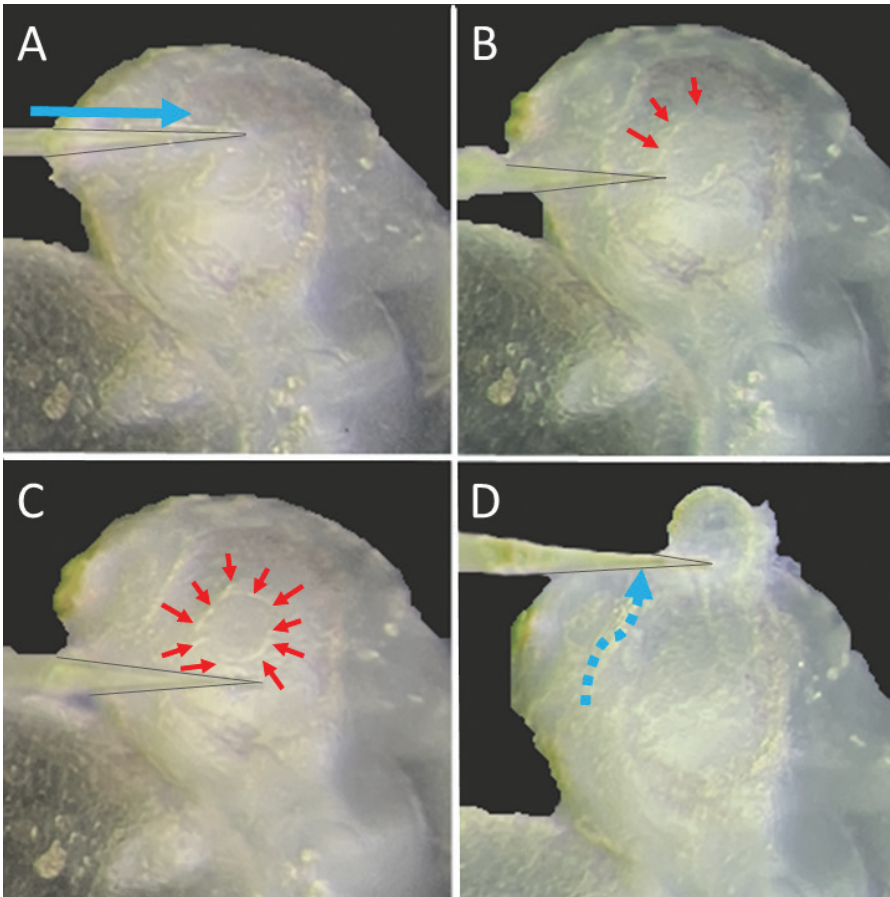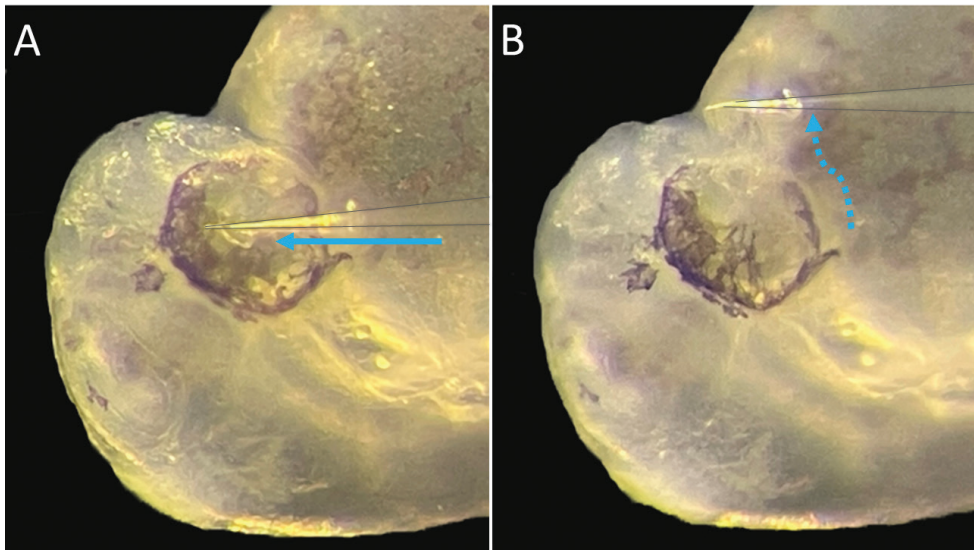
Figure 4. Style #2 for doing lens ablations **A** with the micromanipulator, slowly bring down the needle by the side of the lens and puncture the surface ectoderm. While inside the optic cup, position the needle so that it is under the lens **B** with a coordinated motion of the hand holding the Petri dish and the micromanipulator, move the needle away from the eye with the lens position in the center. The optic cup will distend until it rips open, with the lens bursting out of the eye cup.

(Fig. 3D). It is common for the lens to brake during this step, especially if no liquid has been added above the agar. With the needle, any lens remains can then be scooped out.

Style 2: With the micromanipulator, slowly bring down the needle by the side of the lens and puncture the surface ectoderm. While inserted, position the needle so that it is under the lens (Fig. 4A). Afterward, move the needle away from the eye with the lens positioned in the center. This is done with a coordinated motion of the hand holding the Petri dish and the micromanipulator. The optic cup will distend until it rips open, with the lens bursting out of the eye cup (Fig. 4B). In most cases, the lens will be obliterated. With the needle, any lens remains can then be scooped out. Style #2 is riskier because, depending on the robustness of the specimen and the grip the agarose has on the specimen, if the ripping of the tissue is done too fast, it can dislodge other internal tissues and kill the specimen. If the agar cover is not too deep, the specimen can also detach from the agar before the lens is ripped out. We found that experience and gentle slowness while pulling was necessary to perform this style effectively. Once this style is mastered, time dedicated per lensectomy is shortened without significantly reducing survival.

Style 3: With the micromanipulator, slowly bring down the needle just by the side of the lens, closer than the previous two styles, and puncture the overlying ectoderm. Position the needle so that it is above the lens instead of below (Fig. 5A). Gently scrape the overlaying agarose and scrape the tissue over the lens. The tissue will tear, and the lens will float up if there is liquid (Fig. 5B). It may need some nudging with the needle.

- If lensectomy is to be done on only one side of the face, submerge the agar containing the specimen in embryo medium and gently dislodge the embryo from the agar; with a blunt tip dissection needle made with a thin tungsten wire or the hair of a toothbrush, stroke downward the agar around the embryo starting around the tail. Pieces of agar will dislodge. Progressively dislodge fragments of agar from tail to head until the specimen is released (Fig. 2I). Caution should be used to minimize touching the specimen, especially with the tip of the needle. Alternatively, fine-tip twicers can dislodge agar on either side of the embryo, again, from tail to head. Once free, the embryo should be transferred to clean embryo medium and put in the incubator.

- If lensectomy is done on both sides, it is best to have the agar dry, not over-laid with liquid. With a scalpel, cut a rectangle of the agar around the specimen (Fig. 2D). Very gently slide the scalpel under the rectangle of agar with the specimen. Helped with twicers, flipping around the agar slab, to expose the specimen on the other side (Fig. 2E, F). If the specimen is dislodged from the agar, add embryo medium to keep it under water. Gently flip it around. Flipping around specimens that have dislodged from the agar slab involves directly contacting the specimen and tends to cause more harm.

- Add 2% agarose around the rectangle of agar or over the specimen if it was dislodged (Fig. 2G, H). Caution should be used in not adding too much agar above the head of the specimen. When too deep, the needle gets deflected from its target, and it is difficult to see the structures.

- Repeat the previous steps to dislodge the second lens.
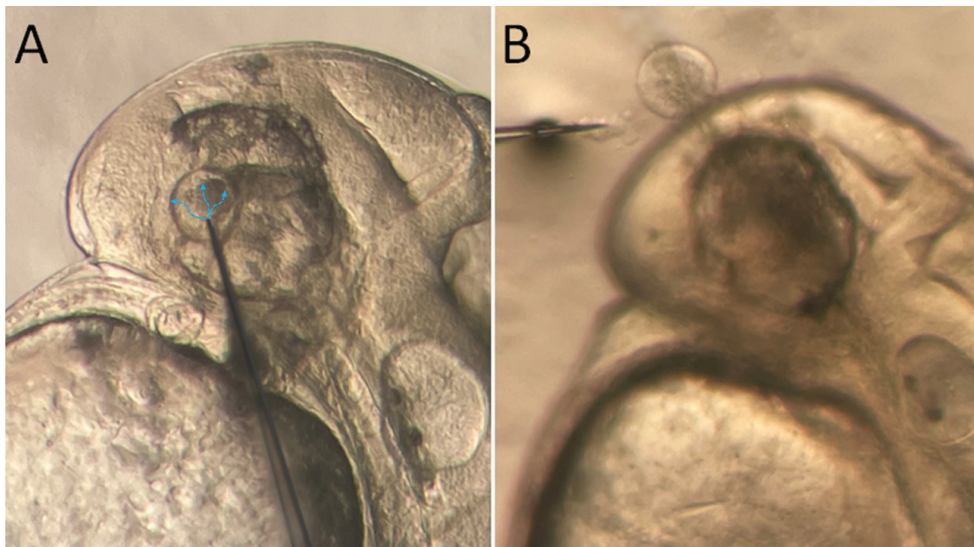
Figure 5. Style #3 for doing lens ablations **A** with the micromanipulator, slowly bring down the needle just by the side of the lens, closer than the previous two styles, and puncture the surface ectoderm. While inside the optic cup, position the needle so that it is above the lens instead of below. Gently scrape the overlaying agarose and scrape the tissue over the lens **B** the tissue will tear, and the lens will float up if there is liquid. It may need some nudging with the needle.

Style #3 works best in younger embryos that have just hatched. In older specimens, the tissue covering the lens has grown, which may require the stronger tearing of styles #1 or #2. Style #3 is the preferred style of lens removal if lenses are to be collected for transplants or other studies.

Part 5: Transplantation procedure

In this case, at the beginning of the protocol, after embedding the specimen in 2% agarose, overlay it with EM containing 1.2% agarose. Use style #3 preferentially. Once free, lenses will float in the medium. Using the blunt needle, carefully push the lens of the donor to just above where the host lens would normally be, and then push it down into the eye with the blunt needle. The host lens may be discarded.

Leave donor and host embryos in 1.2% agarose for 30–60 minutes, then release them from the agarose using the sharp needle and transfer them into EM in the incubator.

Results

Bilateral lensectomies using microinjection needles made from glass capillaries attached to a manual micromanipulator were extremely successful compared to previous results using tungsten needles held by hand (Hélène Hinaux, personal communication; Elipot et al. 2013). Two hundred fifty-six live specimens were obtained from three broods. Of them, 96 underwent lens ablations on both sides, and 160 did not, serving as control siblings. Both the experimental and the control groups were kept under the same conditions. Initial postoperative survival was 100%, as reported in Espinasa et al. (2014) and Blin et al. (2018). In the three broods used for those experiments, no single specimen died during the operation, and all were alive 24 hrs post-operation. After one week post-operation, 88 (91.6%) lensectomized fish were alive, and 158 (98.7%) control fish were alive. After one month, 80 (83.3%) lensectomized and 145 (90.6%) control fish remained. Survival rates were equivalent between lensectomized fish and their siblings on which no operation was performed and kept in the same incubator (P=0.112, Fisher exact test). No impact on non-targeted tissues was seen in both experimental and control group.

Time dedicated to conducting ablations in each brood was about 10 hrs, giving an average of about 3.3 successful double ablations per hour. All treated specimens developed normally and had equivalent body sizes to their untreated siblings. One week after the procedure, individuals on which a single side lensectomy was performed already one significantly smaller eye (Fig. 1E). The success of the lensectomy procedures in triggering eye degeneration was observed by the specimens' significantly regressed eyes compared to untreated specimens of the same brood after a few months of development (Fig. 6).
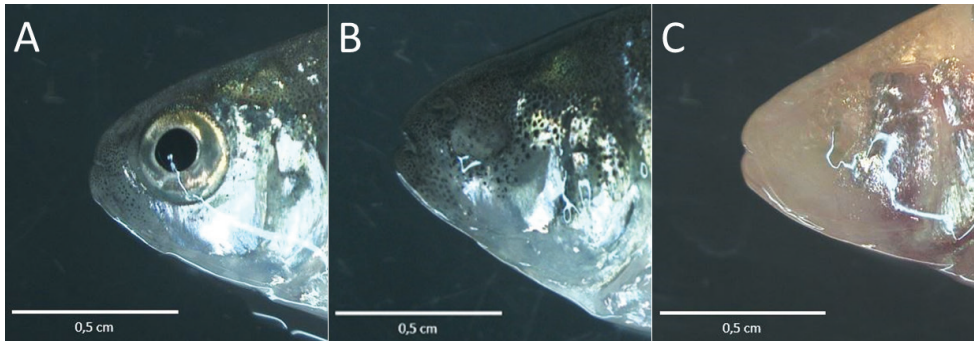
Figure 6. Four month-old *Astyanax mexicanus* **A** non-operated surface fish **B** lensectomized surface fish **C** Pachón cavefish.

Discussion

The technique for embryonic lens removal described for *Astyanax* fish constitutes a significant modification for improvement. It is also readily applicable to zebrafish. Production of healthy individuals with double lensectomies increases by at least an order of magnitude (Hélène Hinaux, personal communication). Hundreds of specimens can now be made available for study, thus solving previous sample size limitations that hindered research on the developmental effects of eye regression.

Compared with previous lens removal techniques performed on *Astyanax* or zebrafish (see video from Zhang et al. 2009, at 1:25–1:35 minutes; https://www.jove.com/fr/v/1258/lens-transplantation-zebrafish-its-application-analysis-eye?section=0&&?section=0&&), it is noticeable the reasons our technique constitutes a significant improvement. As seen in this video, previous techniques that control the needle by hand are very jittery. While the lens has a diameter of only 50 µm, hand shaking makes the needle oscillate and "jump" up to 100 µm. The eye and head of the fish embryo are seen to be pushed broadly and often. The precision for puncturing the epithelium around the lens is very difficult. Puncturing neighboring structures, such as the brain or heart, often happens with the previous technique. On the contrary, with a micromanipulator, movements of the needle can be controlled easily at less than 5 µm. Microinjection needles made from pulled glass capillaries also appear to puncture the tissues more smoothly than previously used tungsten needles.

Several steps require special attention

Healthy conditions for the breeding colony: Survivorship of embryos can be drastically different between laboratories due to the conditions in which parents and the embryos are kept. The technique described here can produce live embryos, with the limiting factor being the general survivorship of embryos within the specific laboratory conditions they are kept.

Clean needles: Throughout the procedure, the needles progressively get covered in what appears to be a fatty substance (Fig. 1D). This "glob" blunts the needle, and instead of easily puncturing the tissue, it pushes down the eye until uncontrolled rupture may happen. Sometimes the needle tip can be cleaned by immersion in the agar and sliding the needle sideways while inside the agar. Nonetheless, it is best to exchange it for a new one after about three lenses have been removed, or when the glob develops.

The agar's depth significantly affects the efficiency of the procedure (Fig. 2C). Especially when doing lens ablation on both sides, one side will be overlaid by more agar. Practice until achieving correct conditions. We found that, as a generality, adding too much agar was a more significant problem.

Gentle, slow motions are to be done with the micromanipulator throughout the process (Fig. 1C). While up to 10 fish were successfully put in a row to undergo the procedure, we found that a slower approach, working with about three fish at a time, was the most efficient (Fig. 2G–I).

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RESEARCH ARTICLE

First data on testate amoebae associated with the endemic cave bivalve *Congeria jalzici* Morton & Bilandžija, 2013 with a description of *Psammonobiotus dinarica* sp. nov.

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Abstract

Testate amoebae are phylogenetically a very diverse group of eukaryotic microorganisms. They can be found in marine and freshwater habitats and in soil. Some of these single-celled organisms inhabit both surface and cave habitats, but their diversity in caves has barely been explored. Recent studies in the Dinaric region imply that testate amoebae in caves show a high diversity. The aim of this study was to identify the alpha diversity of testate amoebae in the Lika region (Dinaric karst, Croatia) and to compare the habitats of different caves based on testate amoebae assemblages. In eight caves we found more than 40 testate amoebae taxa, including a new testate amoeba species, *Psammonobiotus dinarica* **sp. nov.** The greatest diversity of testate amoebae was found in Markov ponor (27 taxa). The Bray-Curtis Similarity Index showed that testate amoebae assemblages in caves inhabited by the endemic and endangered cave bivalve *Congeria jalzici* (Markov ponor, Dankov ponor and Dražice ponor) differ from caves not inhabited by this species. This differentiation is attributed to the impact of the sinking Lika river, which occasionally completely submerges these caves, creating specific habitats for eukaryotic microorganisms. This study contributes to our understanding of the diversity, biogeography and ecology of testate amoebae in caves, as well as providing further insight into the conditions that sustain populations of *C. jalzici*.

Introduction

The Dinaric karst of the Western Balkans is world's most important subterranean biodiversity hotspot and is classified as a unique and rare ecosystem with numerous endemic species (Sket et al. 2004; Culver et al. 2006; Sket 2012). Biodiversity uniqueness has been recognized by a subterranean community that reflect adaptations to specific conditions and past intense geologic events that drove speciation and migration (Culver and Sket 2000).

Among the representatives of stigobionts as aquatic species in Europe, only one genus of bivalves is represented in caves. The Tertiary relict genus *Congeria* currently survives as three distinct species, namely *Congeria kusceri* Bole, 1962, *C. mulaomerovici* Morton & Bilandžija, 2013, and *C. jalzici* Morton & Bilandžija, 2013, with a highly fragmented distribution (Bilandžija et al. 2014; Jovanović Glavaš et al. 2017). All three species occur in only 15 caves in the Dinaric karst region (Jalžić 1998, 2001; Bilandžija et al. 2014; Jovanović Glavaš et al. 2017), but the rapid decline or disappearance of *Congeria* populations observed in recent decades underscores that much more needs to be done for the species conservation (Bilandžija et al. 2021).

Among the many ecological traits of *Congeria* (Morton and Puljas 2013; Puljas et al. 2014), the most interesting is adaptation to extreme habitat variations (presence of water and temperatures). Sinkholes with *Congeria* in the Lika region are completely or partially flooded only during high water events. These flooding events are partly controlled by the operation of the hydropower system on the Lika River. When these sinkholes are flooded, they provide an optimal habitat for *Congeria*, as it allows them to feed and reproduce. After the flooding of the Lika River waters subside, the slow decline of the water level in the sinkholes starts. Habitats that were once running water slowly turn into lake habitats and eventually the water is completely depleted. After that, the water on *Congeria* habitats is limited to seeping water that comes from the epikarst layer above the cave and wet walls. When *Congeria* habitat is converted to non-aquatic habitat, the most extreme conditions for the species begin. The importance of this habitat is usually discussed in the literature as an extreme part of the life cycle of *Congeria* (Jovanović Glavaš et al. 2017; Bilandžija et al. 2021), but there are no further data on the habitat itself. There are many active sinkholes in the Dinaric karst region and many caves with constantly flooded channels, but the distribution of *Congeria* species is limited to just a few localities. This implies that caves with *Congeria* hold some traits that are unique and differentiate them from other caves.

Despite the fact that *Congeria* species are under high threats both due to various human interventions (e.g. hydrotechnical projects) and climate change (Bilandžija et al. 2021), very little data is available on this species. Previous studies of *Congeria* spp. have included some data on species biology and population structure (Jalžić 1998, 2001; Morton et al. 1998; Morton and Puljas 2013; Bilandžija et al. 2014; Puljas et al. 2014; Jovanović Glavaš et al. 2017), evolutionary biology (Stepien et al. 2001; Bilandžija et al. 2014) and viruses in *Congeria* species (Scapolatiello et al. 2022). Relationships of *Congeria* and unicellular eukaryotic microorganisms (protists) have never been investigated. Only data on protists from caves with *Congeria* are findings of algae in the aphotic zone of Markov ponor, Dankov ponor and Dražice ponor, noted by Baković et al. (2022b). Their presence was attributed to flood waters of the Lika River. When discussing cave protists in the context of *Congeria*, we can only make some hypotheteses based on the general ecology of these groups of organisms. Some cave protists could compete with *Congeria* for food (e.g., organic particles, bacteria) (Hausmann et al. 2003) or even represent a compatible food source for *Congeria* (very small eukaryots) (Morton et al. 1998; Gosling 2003). During the high water periods (when *Congeria* colonies are submerged) metabolic products of *Congeria* (urine, faeces) may alter the microenvironment around them, which may affect the composition of microorganisms, but it is expected that this impact will diminish in periods of drought as *Congeria* is in a dormant stage. The most distinctive common trait of cave protists and *Congeria* are habitats they share. For this reason the focus of this paper will be on testate amoebae assemblages present in cave habitats with *Congeria*, as opposed to other caves in the Lika region.

Existing data on cave protists in general are very limited. Caves are inhabited by mostly cosmopolitan protists described from epigean habitats (Gittleson and Hoover 1969, 1970; Golemansky and Bonnet 1994). Nevertheless, several species and forms have been described from caves (Chibisova 1967; Delhez and Chardez 1970; Walochnik and Mulec 2009; Baković et al. 2019) and cave entrances (Main 2003; Bhatt and Karthick 2020; Soler-Zamora et al. 2021). Protists are the trophic link between metazoans and prokaryotic organisms (archaea and bacteria), and thus they are important members of cave ecosystems. Considering their small size, short life cycle, quick response to environmental changes, numerous communities and diverse ecological niches they fill (Hausmann et al. 2003), protists are very compatible as indicator organisms (Foissner and Berger 1996; Bellinger and Siegee 2015). Till now, only ciliates have been investigated in the context of environmental indication in caves by Italian researchers (Coppellotti and Guidolin 1999). Baković et al. (2022a) showed that protist assemblages can be used for identification of habitat diversity in caves. Among protist from caves, great potential for researching cave ecology can be attributed to testate amoebae. Testate amoebae are very diverse in caves in comparison with other protists (Baković et al. 2019) and the species composition is determined by the specific habitats (Golemansky and Bonnet 1994) and cave heterogeneity (Mazei

et al. 2012). They have been recognized and tested as valuable indicators of environmental changes in many surface habitats such as peatlands, soil and aquatic habitats (e.g. Bobrov 2005; Qin et al. 2016; Carballeira and Pontevedra-Pombal 2021). Data on protists in the wider area of the Lika region are scarce. The only data on testate amoebae including caves from this research is that the Čakovac cave, the Horvatova cave and the Pražina cave are inhabited by the recently described species *Centropyxis bipilata* (Baković et al. 2019).

The goal of this study was to identify the diversity of testate amoebae present in selected caves of the Lika region and to determine whether there are differences between testate amoebae assemblages from caves inhabited by the endemic bivalve *C. jalzici* and other caves not inhabited by this species. This research brings the first data on heterotrophic protists associated with *C. jalzici* and the description of a new testate amoeba species for science.

Research location and hydrological settings

All studied caves are presented in Table 1 with cave name and location, sampling date, sampled habitat of testate amoebae and sample designation.

Markov ponor, Dankov ponor and Dražice ponor are sinkhole type caves formed in deposits of Jurasic limestones and dolomites ($J_3^{1,2}$, J_2, J_1^4). They are located on the NE edge of the Lipovo polje (karst field) (elevation approx. 451 m m.a.s.l.) (Fig. 1). These caves are natural sinkholes of the Lika river, but the flooding regime is modified after the building of two artificial dams (the Selište and the dam of the Senj Hydro Power Plant). Water is released in sinkholes just during very high waters. Markov ponor is very complex, multilevel objects, while Dankov ponor and Dražice ponor are less complex objects. Pražina pećina, Buklina, Pećina u Čakovcu and Horvatova špilja are formed in deposits of Eocen-Oligocen breccias-conglomerates (E, Ol) in the elevated area of Mlakva and Mlakvina greda (elevation 521–595m m.a.s.l.), while Samograd is formed in limestone deposits of the Upper Cretaceous, Cenoman and Turone in the Grabovača area (670 m m.a.s.l.) (Fig. 1). Horvatova špilja and Pećina u Čakovcu are multichannel caves. The Pražina pećina and Buklina caves are of a simple morphology. In the lower levels of Pećina u Čakovcu a cave lake is present. Horvatova špilja is located in the artificial dam of the Senj Hydro Power Plant and it is partially and occasionally flooded. In Pražina pećina and Buklina only seeping and dripping water is present. Total cave channel lengths are: Dankov ponor=250 m, Dražice ponor=approx. 140 m, Markov ponor=2557 m, Pražina pećina=27 m, Buklina=20 m, Pećina u Čakovcu=267 m, Samograd=345 m and Horvatova špilja=500 m. In the wider area of these caves building of new hydropower accumulation is planned in the future. It will completely flood the Pećina u Čakovcu and further modify the hydrological regime of Markov ponor, Dankov ponor and Dražice ponor by decreasing flooding frequency and quantities of water flooding these caves (Stroj 2010; Elektroprojekt Ltd. 2017).
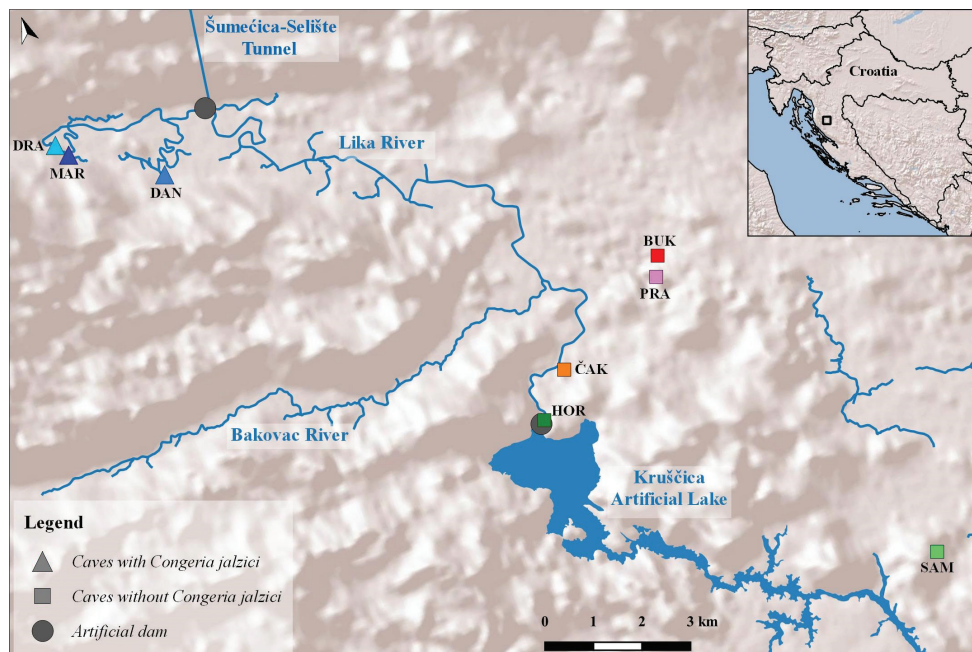
Figure 1. Research location and hydrographic map showing the main permanent and temporary water bodies and the hydrotechnical tunnel (DAN – Dankov ponor, DRA – Dražice ponor, MAR – Markov ponor, BUK – Buklina, PRA – Pražina pećina, ČAK – Pećina u Čakovcu, HOR – Horvatova pećina, SAM - Samograd).

Methodology

Description of studied habitats

Caves with *Congeria jalzici* (Markov ponor, Dankov ponor, Dražice ponor) are seasonally completely or partially flooded by the Lika river that sinks into these caves. Thus, habitats inside caves are transforming depending on the hydrological regime. These caves were studied during periods of low waters, when the caves were accessible to speleobiologists. Studied habitats were: a small cave lake with submerged colonies of *C. jalzici*, cave walls with attached individuals of *C. jalzici*, cave walls without individuals of *C. jalzici* and small cave pools unrelated to *C. jalzici*. The water present in a small cave lake with submerged colonies of *C. jalzici* is a remnant of water imported to the cave by the Lika river during the flood events. Organic debris and small waste (plastic etc.) fragments were present on the surface of the lake. The water level in these lakes gradually drops during the dry season, leaving the colonies of *C. jalzici* out of the water. Habitats, with attached *C. jalzici*, outside the water column are transitional habitats (hygropetric) and wet walls. Transitional habitats (hygropetric) are very thin and consist of slowly seeping water that flows over the surface of the cave wall and attached individuals of *C. jalzici*. Although there is no visible seeping water on the wet

walls, they are still permanently wet due to the high air humidity inside the cave. There are also several locations with small pools in the caves. The distinction between transitional habitats and wet walls is not always clear. The investigated sinter and clay pools in these caves are relatively small (a few centimeters wide and deep).

The investigated habitats in the caves without *C. jalzici* (Pražina pećina, Buklina, Pećina u Čakovcu, Horvatova špilja and Samograd) were not flooded by exogenous rivers. These researched habitats depended on seeping and dripping water. In most habitats of these caves traces of old bats guano are present, which is detectable in microscopical samples by the presence of small quantities of insects leftovers. Only in samples BUK_th and SAM_sp significant quantities of bats guano were found, while sample HOR_bg consisted of bats guano deposits (Table 1). Transitional habitats (hygropetric) in these caves were characterized by a very thin layer of seeping water that flows on the surface of the cave walls. The sinter pools in Pražina pećina and Samograd were 30 cm wide and up to 15 cm deep, while the sinter pools in Pećina u Čakovcu were several cm wide and deep.

Table 1. Collected samples.

No.	Cave name and location	Sampling date	Sampled habitat of testate amoebae	Sample designation
Caves with Congeria jalzici				
1	Dankov ponor, Lipovo polje, CRO	08/2016*	Transitional habitat with *C. jalzici*	DAN_th
2	Dankov ponor, Lipovo polje, CRO	08/2016	*Ex situ* swabs of shells of living *C. jalzici*	DAN_esw
3	Dražice ponor, Lipovo polje, CRO	08/2016*	Transitional habitat with *C. jalzici*	DRA_th
4	Dražice ponor, Lipovo polje, CRO	11/2018	Transitional habitat	DRA_th2
5	Dražice ponor, Lipovo polje, CRO	11/2018	Transitional habitat	DRA_th3
6	Dražice ponor, Lipovo polje, CRO	11/2018	*In situ* swabs of shells of living *C. jalzici*	DRA_isw
7	Dražice ponor, Lipovo polje, CRO	11/2018	*Ex situ* swabs of shells of living *C. jalzici*	DRA_esw
8	Markov ponor, Lipovo polje, CRO	09/2016*	Transitional habitat with *C. jalzici*	MAR_th1
9	Markov ponor, Lipovo polje, CRO	09/2016*	Transitional habitat with *C. jalzici*	MAR_th2
10	Markov ponor, Lipovo polje, CRO	10/2016	Plankton from cave lake (65 µm mesh)	MAR_pla1
11	Markov ponor, Lipovo polje, CRO	10/2016	Plankton from cave lake (120 µm mesh)	MAR_pla2
12	Markov ponor, Lipovo polje, CRO	10/2016	Floating debris from cave lake	MAR_deb
13	Markov ponor, Lipovo polje, CRO	11/2021	Transitional habitat	MAR_th1
14	Markov ponor, Lipovo polje, CRO	11/2021	Transitional habitat	MAR_th2
15	Markov ponor, Lipovo polje, CRO	11/2021	Sinter pool filled with water	MAR_sin
16	Markov ponor, Lipovo polje, CRO	11/2021	Clay pool	MAR_clp
Caves without Congeria jalzici				
17	Buklina (syn. Špilja u Poljani), Poljana, CRO	08/2016*	Transitional habitat	BUK_th
18	Horvatova špilja, HPP Sklope, Mlakva, CRO	08/2016*	Bats guano deposits	HOR_bg
19	Horvatova špilja, HPP Sklope, Mlakva, CRO	08/2016*	Transitional habitat	HOR_th
20	Pećina u Čakovcu, Bobići, Mlakva, CRO	08/2016*	Transitional habitat	ČAK_th1
21	Pećina u Čakovcu, Bobići, Mlakva, CRO	08/2016*	Terrestrial sediment	ČAK_terr
22	Pećina u Čakovcu, Bobići, Mlakva, CRO	09/2016*	Transitional habitat	ČAK_th2
23	Pećina u Čakovcu, Bobići, Mlakva, CRO	09/2016*	Sinter pool filled with water	ČAK_sp
24	Pražina pećina, Poljana, Mlakva, CRO	08/2016*	Transitional habitat	PRA_th
25	Pražina pećina, Poljana, Mlakva, CRO	08/2016*	Sinter pool filled with water	PRA_sp
26	Samograd, Grabovača, Perušić, CRO	10/2016*	Transitional habitat	SAM_th
27	Samograd, Grabovača, Perušić, CRO	10/2016*	Sinter pool filled with water	SAM_sp

Samples marked with asterisk (*) were used for a descriptive statistics; other samples were used exclusively for the description of alpha diversity of testate amoebae.

Sampling

The samples from Markov ponor (MAR_th1, MAR_th2), Dankov ponor (DAN_th) and Dražice ponor (DRA_th) (Table 1) were collected by gently scrubbing with a plastic brush the surfaces of shells of living *Congeria jalzici* and the transitional habitats on which they were attached. The brush was then washed in a plastic container filled with 40 ml of tap water. The process was repeated till the water became muddied. Selected samples from Markov ponor (MAR_th1, MAR_th2, MAR_sin, MAR_clp) and Dražice ponor (DRA_th2, DRA_th3), and all samples from the caves Pražina pećina, Pećina u Čakovcu, Horvatova špilja and Samograd (Table 1) were collected by using the methodology presented by Baković et al. (2019).

Plankton samples from Markov ponor (MAR_pla1, MAR_pla2) were collected using plankton nets (65 μm and 120 μm mesh size) and placed into plastic containers. Floating debris from the cave lake (MAR_deb) was handpicked and placed into a plastic container filled with lake water (Table 1).

Samples of protists living *in situ* on the outer shells of *C. jalzici* (DRA_isw) were collected by gently scrubbing with a plastic brush exclusively on the surfaces of shells of living *C. jalzici* on transitional habitats. The brush wa then washed in a plastic container filled with 40 ml of tap water. The process was repeated until the water became cloudy. *Ex situ* samples of protists living on the outer shells of *C. jalzici* (DRA_esw, DAN_esw) were scrubbed gently with a microscope cover slip (Table 1). Scrubbed material was transferred directly to a microscope slide. One drop of water was added to the microscope slide before the examination.

Transport to the lab, maintaining the temperature, examination

Samples were transported to the laboratory within several hours after the sampling and stored at a temperature of 4–8 °C and analyzed within 48h from the time of the collection. Exceptions were selected samples from Markov ponor (MAR_pla1, MAR_pla2, MAR_deb) (Table 1) which were stored at room temperature and examined within a 72 hour time period from the time of collection.

Triplets of 0.2 ml from each sample (total of 0.6 ml) were examined using a Carl Zeiss Axiostar microscope with 100, 400 and 1000 times magnification. For additional species examination a Zeiss Axioscop 40 FL and an Olympus BX51 microscope with Phase Contrast and Differential Interference Contrast (DIC) were used. A Nikon Diaphot inverted microscope was used for examining samples and isolating specimens. Adobe Photoshop and ToupView software were used for image processing and measurements. All testate amoebae were identified to the lowest possible level. Species were identified by using the following literature to begin with: Golemansky and Todorov (2004), Mazei and Tsyganov (2006), Nicholls (2005), Ogden and Hedley (1980), Siemensma (2021) and Todorov and Bankov (2019). An abundance of testate amoebae is noted only for samples no. 1–2, 5–6, 14–24. Counted were all individuals in the examined volume (0.6 ml). Based on the number obtained, it was estimated (mathematical calculation) how many individuals would be in 1 ml. Since literature data on testate amoebae abundance in caves

are lacking, available data from this research were used to determine ranges (low/high abundance). Samples used only for the studding testate amoebae diversity were intensively examined in search of testate amoebae tests (> than 0.6 ml of sample was examined).

Data analyses

Descriptive statistics (Bray-Curtis similarity, Shannon diversity index and Pielou's evenness index) was done in Primer 6 (PRIMER-e Ltd) on selected samples collected in 2016 (samples no. 1–2, 5–6, 14–24) which represented comparable data. Other samples (3, 4, 7, 8, 9, 10, 11, 12, 13) were used only for the contribution to knowledge on testate amoebae diversity.

Results

Taxa diversity

Testate amoebae were found in 23 samples (85.1%). Exceptions were the samples of the cave lake plankton (MAR_pla1, MAR_pla2) and in *ex situ* swabs from the shells of *C. jalzici* (DAN_esw, DRA_esw) that did not reveal any testate amoebae. Over forty testate amoebae taxa were distinguished (Table 2, Plates 1–3). Among them, 33 taxa have been identified to the species level, while others have been identified to the genus level. Several taxa were identified just as testate amoebae due to the small number of observed individuals that prevented accurate identification. Particularly diverse were taxa of the genera *Centropyxis* and *Difflugia*. The highest diversity of taxa (27) was found in Markov ponor. Among the taxa found during this research, one new species for science was identified and further described in this research – *Psammonobiotus dinarica* sp. nov.

Taxonomic description

Phylum: Cercozoa Cavalier-Smith 1998, emend. Adl et al. 2005; emend. Cavalier-Smith 2018
Class: Thecofilosea Cavalier-Smith 2003, emend. Cavalier-Smith 2011
Family: Incertae sedis Psammonobiotidae Golemansky 1974, emend. Meisterfeld 2002
Genus *Psammonobiotus* Golemansky, 1967

Psammonobiotus dinarica **Baković & Siemensma, sp. nov.**
https://zoobank.org/89057128-7C93-434D-BA9E-A0CF8F4E7FAB
Plates 2F, 3A–E

Diagnosis. Shell is bilaterally symmetrical, in dorsal and ventral views spherical to ovoid in outline and in lateral view compressed with a length/height ratio of about 2.3. A funnel-shaped collar extends from a kidney-shaped oral aperture. In lateral view,
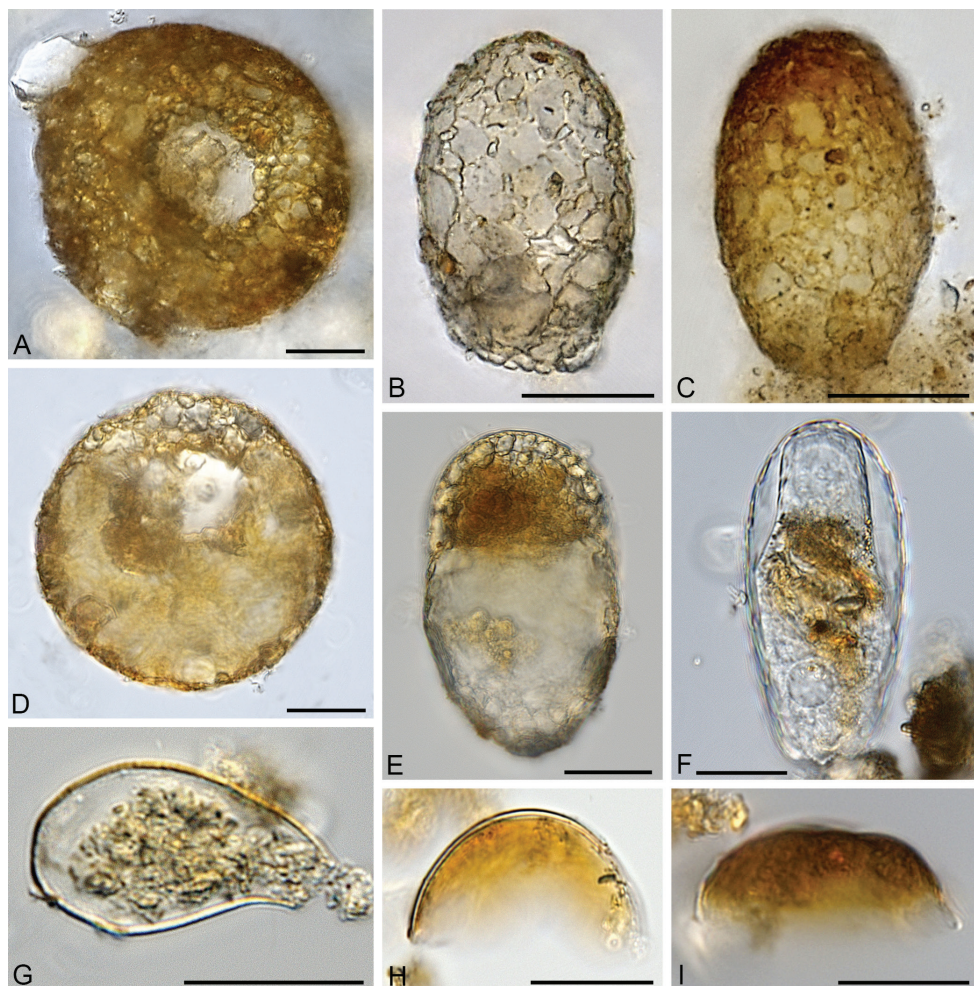
Plate 1. A *Cyclopyxis kahli* **B** *Difflugia* cf *pristis* **C** *Pseudodifflugia gracilis* **D** *Centropyxis aerophila* **E** *Centropyxis constricta* **F** *Euglypha tuberculata* **G** *Cyphoderia laevis* **H, I** *Microchlamys patella*. Scale bars: 20 μm.

the angle of the plane of this pseudostome collar is usually zero degrees, but can some-times be as high as 33°. The translucent and fragile organic shell is covered with small irregularly-shaped thin and flat quartz particles. Larger particles are located on the dorsal and distal part of the shell and smaller particles on the ventral side. The rim of the collar is covered with relatively large flat particles. The organic matrix is colorless to dark brown. Length including the collar 45–54 μm; main body width 26–30 μm, height 17–30 μm; collar 20–29 μm across (n=6).

Etymology. The specific name refers to the area where the species was found, the Dinarides or Dinaric Alps, Latin: *dinarica*, a mountain range in, among others, Croatia and Bosnia and Herzegovina.

Type material. Three slides, one with the ***holotype*** and two with ***paratypes***, were mounted in HYDRO-Matrix on glass slides and deposited in the collection of the

Croatian Biospeleological Society under accession numbers TAM4 (holotype), TAM5 and TAM6 (paratypes).

Type locality. Croatia, Lika region, Lipovo polje, Dražice ponor, 44°46'20.2"N, 15°11'10.6"E, 10 November 2018, H. Bilandžija leg.

Differential diagnosis. There are several testate amoebae similar in shape and size to *P. dinarica*: *Centropyxis platystoma*, *Psammonobiotus communis*, *P. septentrialis* and *P. minutus*, some *Centropyxiella* and *Corythionella* species, and *Conicocassis pontigulasiformis*. *Centropyxis platystoma* was described by Penard in 1890, but in 1902 he considered this species identical to Leidy's *C. constricta* (Penard, 1890, 1902). Since he originally described it as *Difflugia platystoma*, the shell must have looked to him as that of a *Difflugia*, with a dense covering of quartz particles. Penard described the shell as "pierreuse", stony. This is different from *P. dinarica*, where the shell is covered with tiny flat particles. In the original description, Penard showed a drawing of the visor with a strongly inwardly curved edge, in contrast to the edge of the collar of *P. dinarica* which is not curved. *P. dinarica* can be distinguished from *P. septentrialis* and *P. minutus* by its larger size, 45–54 μm long versus 10–12 μm and 23–30 μm, respectively (Golemansky 1970; Chardez 1977a). The size is about the same as *P. communis*, 45–54 μm (Golemansky 1967) versus 33–52 μm, but *P. dinarica* can be distinguished from the latter by the kidney-shaped aperture. *P. dinarica* also bears some resemblance to *Conicocassis pontigulasiformis*, but this species is much larger, 82–90 μm, and the test wall has a much more pronounced granular organic cement and a circular aperture (Nasser and Patterson 2015). *P. dinarica* can be distinguished from *Centropyxiella arenaria* by its kidney-shaped aperture, and from *C. elegans* and *C. lucida* by its smaller size, length 45–54 μm versus 70–80 μm and 70–81 μm, respectively (Golemansky and Todorov 2007). It can be distinguished from similarly shaped *Corythionella* species (Golemansky 1970b) by the presence of small elliptical or oval idiosomes in the latter genus.

Biogeography and habitats. *Psammonobiotus dinarica* was found in the Dinaric karst of Croatia and in Bosnia and Herzegovina, in the caves Dražice ponor, Markov ponor (both Lipovo polje, CRO) (Fig. 1, Table 1), Jopićeva cave-Bent system (Krnjak, CRO) and Matešićeva-Popovačka cave system (Slunj, CRO) (Baković N., unpublished data). One empty shell was found in a small karst spring near the Jopićeva cave-Bent system (Krnjak, CRO) (Baković N., unpublished data). This species was also reported in the Velika Bukovačka and the Listvača cave (both in Bosnia and Herzegovina) (Baković et al. 2019). The altitude of these localities ranges from 210 to 950 m.a.s.l. The species inhabits surface sediments of permanent cave streams (dominant), sediments of small cave standing waters (sinter pools and clay pools) and transitional habitats (hygropetric). Only one observation of *P. dinarica* on the shell of a living *Congeria jalzici* was noted.

The following ranges of physio-chemical parameters were present in the habitat of this species: water temperature 5.4–10.7 °C, pH 7.62–8.11, conductivity 161–338 μS/cm. It was only occasionally present in all investigated habitats, but always at low densities (up to 3.3 ind. in 1 ml of aquatic sediment).

Table 2. Taxa diversity.

Taxa	Location							
	DAN	DRA	MAR	BUK	ČAK	HOR	PRA	SAM
1 *Centropyxis aculeata* Ehrenberg, 1838			+					
2 *Centropyxis aerophila* Deflandre, 1929		+	+		+			+
3 *Centropyxis constricta* (Ehrenberg, 1841) Penard, 1890			+					
4 *Centropyxis bipilata* Baković, Siemensma et. al, 2019					+	+	+	
5 *Centropyxis elongata* (Penard) Thomas, 1959			+			+		
6 *Centropyxis laevigata* Penard, 1890		+			+			
7 *Centropyxis plagiostoma* Bonnet et Thomas, 1955			+					
8 *Cryptodifflugia oviformis* Penard, 1902			+	+		+		+
9 *Cyclopyxis eurystoma* Deflandre, 1929		+	+		+	+		
10 *Cyclopyxis kahli* Deflandre, 1929			+					
11 *Cyclopyxis* sp 1					+			
12 *Cyclopyxis* sp 2							+	
13 *Cyphoderia ampulla* (Ehrenberg, 1840) Leidy, 1878			+				+	
14 *Cyphoderia laevis* Penard, 1902			+					
15 *Cyphoderia* sp.			+					
16 *Difflugia lithophila* Penard, 1902		+					+	
17 *Difflugia lucida* Penard 1890		+						
18 *Difflugia* cf. *pristis* Penard, 1902		+	+					
19 *Difflugia oblonga* Ehrenberg, 1838			+					
20 *Difflugia penardi* Hopkinson, 1909		+						
21 *Difflugia pulex* Penard, 1890		+	+					
22 *Difflugia* sp 1		+	+					
23 *Difflugia* sp 2					+		+	
24 *Difflugia* sp 3							+	
25 *Euglypha* sp.			+					+
26 *Euglypha laevis* Perty, 1849			+			+		
27 *Euglypha rotunda* Wailes & Penard, 1911			+	+		+	+	
28 *Euglypha tuberculata* Dujardin, 1841			+					
29 *Heleopera petricola* Leidy, 1879		+					+	
30 *Heleopera* sp.						+		
31 *Heleopera sylvatica* Penard, 1890					+			
32 *Microchlamys patella* (Claparède et Lachmann, 1859) Cockerell, 1911	+	+	+		+			
33 *Paraquadrula irregularis* (Archer, 1877) Deflandre, 1932					+	+		
34 *Parmulina* sp.			+					
35 *Phryganella paradoxa* Penard 1902		+						
36 *Plagiopyxis declivis* Bonnet, 1955			+					
37 *Psammonobiotus dinarica* sp. nov.		+	+					
38 cf *Psammonobiotus linearis* Golemansky, 1971		+						
39 *Pseudodifflugia gracilis* Schlumberger, 1845		+	+					
40 Testacea sp 1		+						
41 Testacea sp 2		+			+			
42 Testacea sp 3		+						
43 Testacea sp 4					+			
44 *Tracheleuglypha dentata* (Vejdovsky, 1882) Deflandre, 1928			+	+		+		+
45 *Trinema enchelys* Ehrenberg, 1838			+					
46 *Trinema lineare* Penard, 1890		+	+		+	+	+	+
Total	1	19	27	3	12	10	9	5

Locations: DAN – Dankov ponor, DRA – Dražice ponor, MAR – Markov ponor, BUK – Buklina, PRA – Pražina pećina, ČAK – Pećina u Čakovcu, HOR – Horvatova pećina, SAM - Samograd.
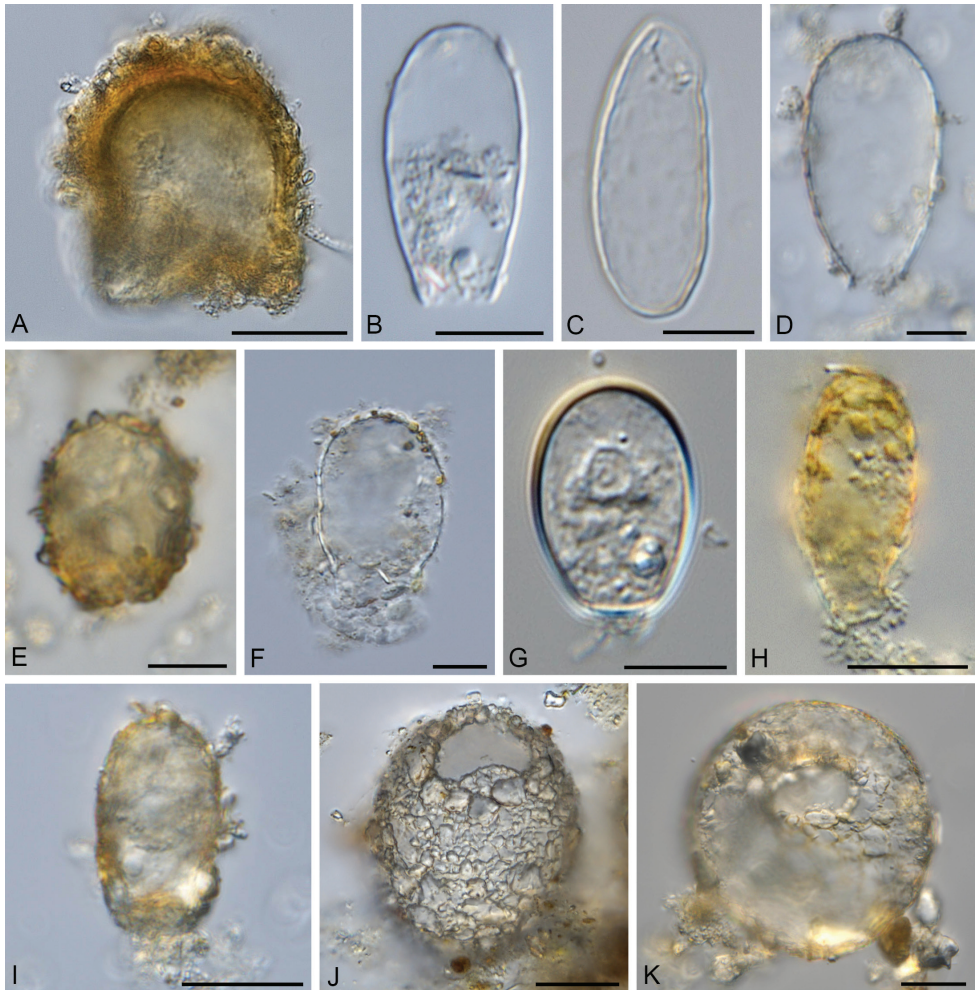
Plate 2. A *Parmulina* sp. **B** *Euglypha laevis* **C** *Trinema lineare* **D** *Tracheleuglypha dentata* **E** *Phryganella paradoxa* **F** *Psammonobiotus dinarica* sp. nov. **G** *Cryptodifflugia oviformis* **H** Testacea sp 1 **I** Testacea sp. 2 **J** Testacea sp. 3 (stacked image) **K** *Centropyxis laevigata*. Scale bars: 20 μm (**A, I–K**); 10 μm (**B–H**).

Remarks. The genus *Psammonobiotus* contains nine beach sand-dwelling species, six recorded only from marine and brackish waters (Golemansky and Todorov 2004), one exclusively from freshwater (*P. dziwnowi*) (Nicholls 2005) and two also from marine and freshwater bodies (*P. communis* and *P. linearis*) (Golemansky and Todorov 2004). *P. dinarica* is the second *Psammonobiotus* species to be reported exclusively from a freshwater biotope. All species known to date have been collected from the psammon from beaches of marine water bodies and some large freshwater lakes (Golemansky and Todorov 2005). The presence of *P. dinarica* in a (freshwater) cave system can therefore be called surprising. However, it is not the first *Psammonobiotus* species to have been found in another ecosystem. The website arcella.nl reports the presence of *Psammonobiotus*
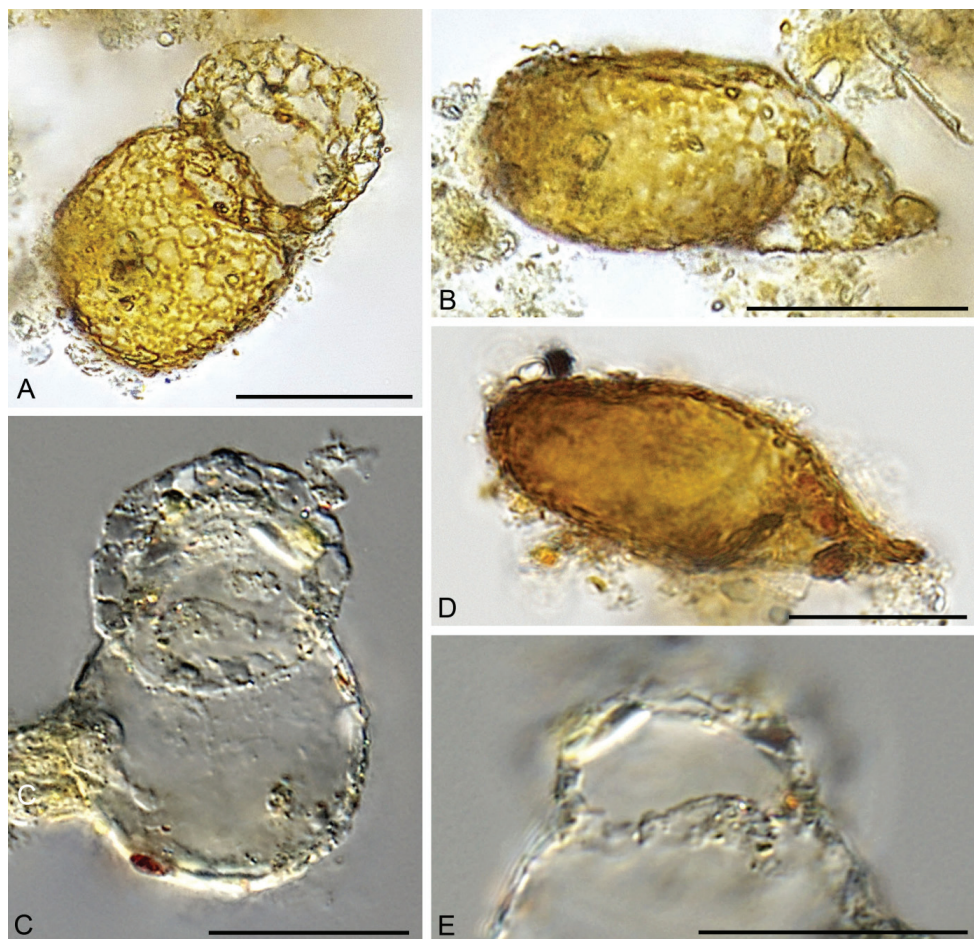
Plate 3. *Psammonobiotus dinarica* **A** shell in ventral view (type specimen) **B** shell in lateral view (type specimen) **C** shell in ventral view **D** shell in lateral view **E** kidney-shaped aperture. Scale bars: 20 μm. **A–D** stacked photomicrographs.

species in a relatively small lake, in organic sediment of a small shallow freshwater ditch, in organic sediment of an isolated pond and in the soil of a peat bog, all in the Netherlands (Siemensma 2021). It shows that our knowledge about the presence and distribution of *Psammonobiotus* species in freshwater and soil biotopes is still very limited.

Regarding the presence of light, all species of the genus *Psammonobiotus* primarily inhabit aphotic biotopes – cave sediments (*P. dinarica*) and interstitial sand habitats (all other species) (Golemansky and Todorov 2005).

Although living specimens of *P. dinarica* have been observed, pseudopodia could never be observed as these specimens were always firmly attached to sediment particles. The brown color, if present, disappears rapidly when the shell is embedded in HYDRO-Matrix.

Testate amoebae assemblages

Comparison of taxa diversity (Fig. 2) showed that most diverse were the sample of bats guano (HOR_bg) and one sample from a sinter pool (ČAK_sp) where guano was not present. Diversity and abundance of testate amoebae in samples from caves inhabited by *C. jalzici* did not show any specific deviation – they vary from low to medium diversity and abundance of taxa.

The highest taxa abundance (Fig. 2) was detected in the sample of bats guano HOR_bg (74 ind. in 1 ml) and two other samples rich in bats guano: BUK_th (55 in. in 1 ml) and SAM_sp (41 ind. in 1 ml). Despite the high taxa diversity, samples from Markov ponor showed relatively a low abundance of taxa (<25 ind. in 1 ml). The lowest abundance was detected in samples of ČAK_th2 (6 ind. in 1 ml), DAN_th, DRA_th, ČAK_sp, HOR_th (all 12 ind. in 1ml) and PRA_th (13 ind. in 1 ml).

Regarding the abundance of individual testate amoebae in caves inhabited by *C. jalzici*, the species that can be highlighted is *Microchlamys patella*. It was present in all the samples from these caves and it was relatively abundant - reaching 5–10 ind. in 1 ml (average 7.08 ind. in 1 ml). In comparison, the abundance of six other testate amoebae present in these caves was 0–3.3 ind. in 1 ml (average 0.41–1.25 ind. in 1 ml). Also, they were not present in all the samples.

Shannon diversity and Pielou's evenness (Fig. 3) did not show specific difference between caves inhabited with *C. jalzici* and other caves. The differences rather varied depending on the individual samples. Highest Shannon index values were in samples MAR_th1 (3.51), ČAK_sp (3.08) and HOR_bg (3.07). The Pielou's evenness was higher in samples ČAK_sp (0.97), ČAK_th2 and DAN_th (both 0.96). The Shannon diversity and Pielou's evenness for the samples DAN_th and SAM_th were zero due to the only one species present in the samples.

The differences between caves inhabited with *C. jalzici* and other caves were well visible in Bray-Curtis similarity dendrogram based on diversity and abundance of the testate amoebae (Fig. 4). Three main branches are distinguished: samples from caves inhabited with *C. jalzici* (MAR_th1, MAR_th2, DAN_th, DRA_th), samples with significant presence of bats guano (BUK_th, HOR_bg, SAM_sp) and other samples from caves uninhabited by *C. jalzici* (all other). Among third branch it could be emphasized higher similarity of sample of terrestrial cave habitat (ČAK_terr) with transitional habitats (ČAK_th1, HOR_th, SAM_th) in contrast to branches containing sinter pools (small aquatic habitats).

Discussion

This research contributes with the first data on testate amoebae inhabiting three caves with the endemic and endangered cave bivalve *Congeria jalzici*, as well as with data from five other caves in the Lika region. Species found in caves in this study are mostly eurybiotic species also present in surface habitats, which is in accord with literature data (Mazei et al. 2012). Among the recorded species is one known only
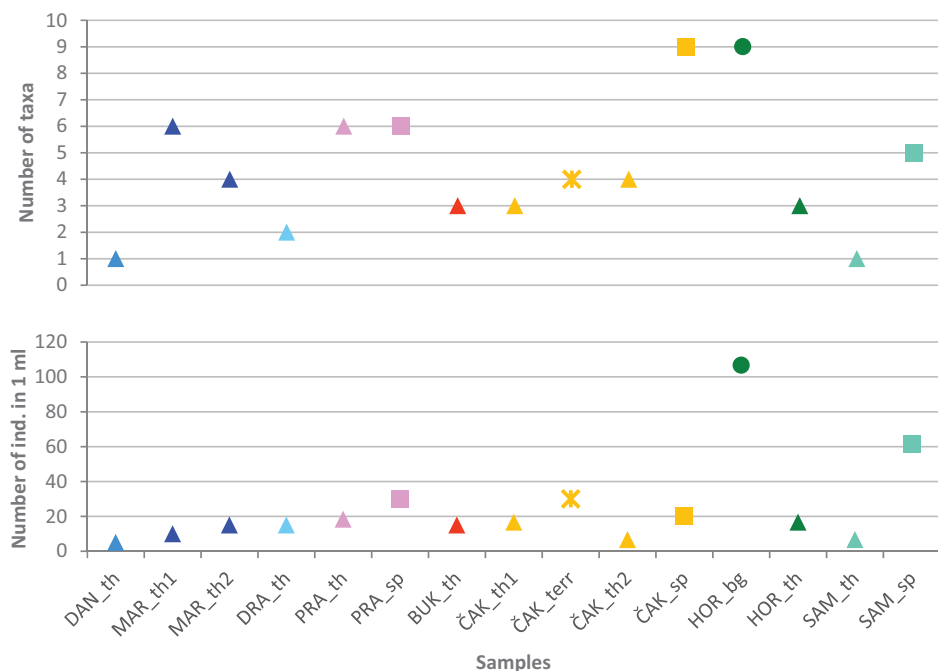
Figure 2. Diversity and abundance of testate amoebae (marker shape: triangle – transitional habitats, asterisk – terrestrial habitats, circle – bats guano deposits, square – sinter pools; marker color: represent different caves).

from caves (*Centropyxis bipilata*) (Baković et al. 2019, 2022a) and one new to science (*Psammonobiotus dinarica* sp. nov.). Species and forms known exclusively from caves (Mazei et al. 2012; Baković et al. 2019; Soler-Zamora et al. 2021), imply that caves may harbor even more species new to science that have been confused with eurybiotic species or have been overlooked (especially in understudied regions).

Despite the fact that an empty shell of *P. dinarica* sp. nov. has been found once in a small karst spring near Jopićeva cave-Bent system (unpublished data, N. Baković), it does not prove beyond doubt that this species also occurs in surface waters, as it could easily be washed away from the subterranean habitats. The full extent of its habitats requires further study. As one individual of *P. dinarica* was found on the shell of a live bivalve *C. jalzici*, this raised the question of whether they were somehow connected. There are many records of epibiotic ciliates associated with cave animals (Matjašič 1962; Dovgal and Vargovitsh 2010) together with some protists that are animal parasites (Gittleson and Hoover 1969), but there is currently no data to support that *P. dinarica* is in some way related to *C. jalzici* in terms of interspecies relations.

The comparison of the testate amoebae diversity in the researched caves was limited due to the small number of samples examined and different time points in which they were collected. Still results from 27 testate amoebae from Markov ponor (from only nine samples) imply that their diversity in individual caves may be even higher than previously reported from Vjetrenica cave in Bosnia and Herzegovina (25 spe-
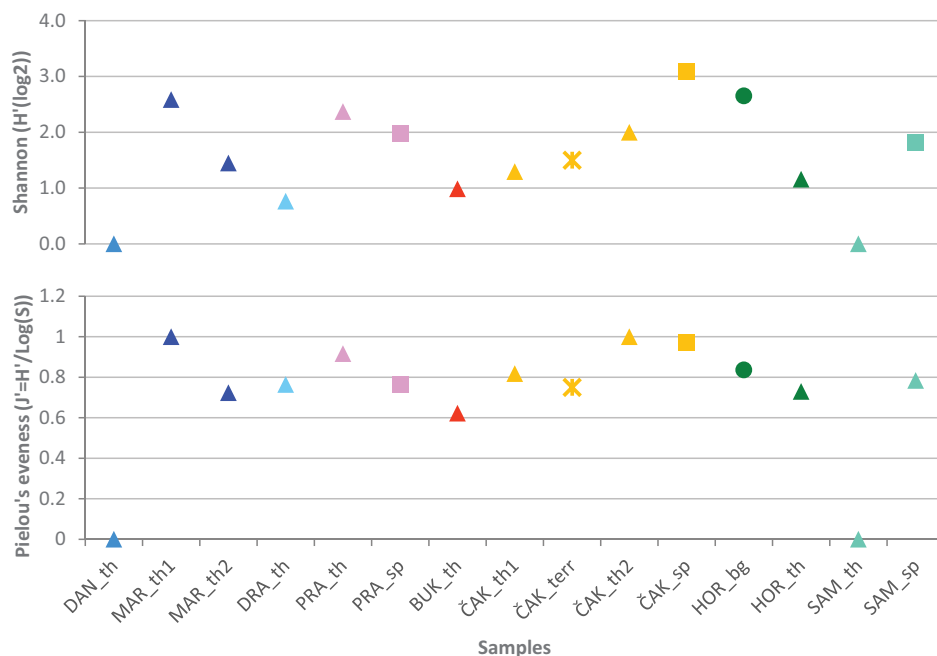
Figure 3. Diversity indices based on testate amoebae assemblages (marker shape: triangle – transitional habitats, asterisk – terrestrial habitats, circle – bats guano deposits, square – sinter pools; marker color: represent different caves).

cies) (Ozimec et al. 2021) and Covo della Guera in Italy (21 species) (Mazei et al. 2012). Nevertheless, data from Baradla Cave (Hungary), where 45 species were found by several researchers (Bereczky 1970), imply that the diversity of testate amoebae in caves could be much higher. The high diversity of Markov ponor may be partly due to flooding by the Lika river. These events could import some species from the surface streams into cave. The comparative study of testate amoebae inside the caves and the riverbeds of sinking streams is completely missing in the literature data. The results on taxa diversity, abundance (Fig. 2) and diversity indices (Fig. 3) from this research were not inconclusive regarding the separation of caves inhabited by *Congeria jalzici* from other caves. The differences between samples can be regarded as differences between habitats and the presence of bats guano that have already been noted by other authors (e.g. Golemansky and Bonnet 1994). When guano is present in caves, it has multiple effects. It increases the suplly of nutrients, which boosts the diversity and abundance of microorganisms, lowers the pH of habitats (Mulec et al. 2015) and provides a more heterogeneous habitat for microorganisms (Baković et al. 2022a).

The main result of this research is the distinction between samples from caves inhabited by cave bivalve *C. jalzici* and other investigated caves based on Bray-Curtis similarity (Fig. 4). The caves inhabited by *C. jalzici* are seasonally completely or partially flooded by the exogenous Lika river (for several weeks or even months). These
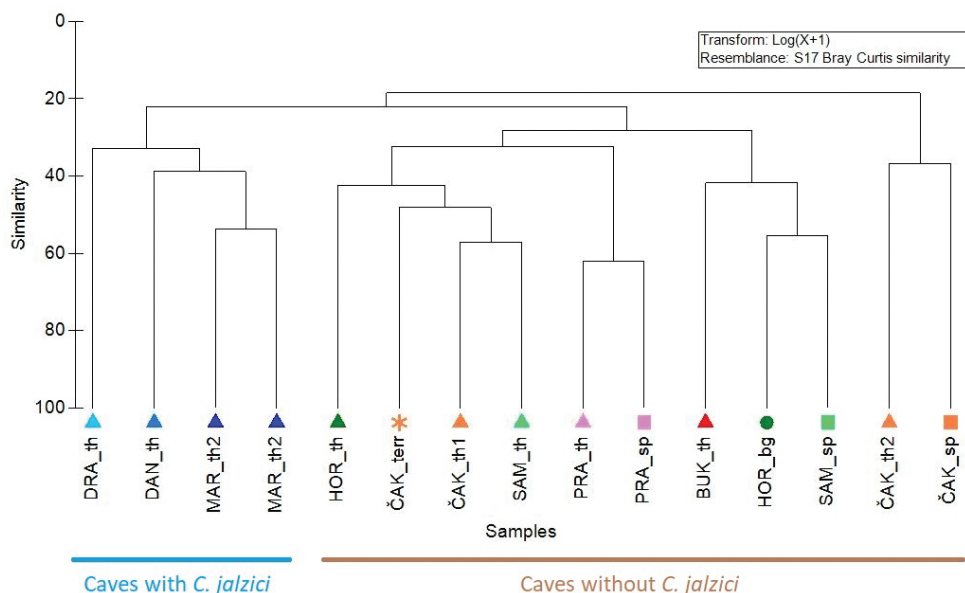
Figure 4. Bray-Curtis similarity dendrogram (marker shape: triangle – transitional habitats, asterisk – terrestrial habitats, circle – bats guano deposits, square – sinter pools; marker color: represent different caves).

flood events mark nutrient-rich periods in these caves as the Lika river imports organic matter into cave habitats that are usually poor in organic matter (Simon et al. 2007). Even after the floodwaters are drained, large amounts of organic material remain in the cave (e.g. water left in cave pools, wood debris). The second way of introduction of surface waters into the wider area of these caves is by water runoff (water losses) through the Lika and Bakovac riverbeds and existing accumulation (artificial lake) Kruščica due to the karstification of this area (Pavičić 1997), a phenomenon that is very common in karst areas (Bonacci 1987; Bonacci and Rubinić 2009). Thus, the impact of surface waters of the Lika and Bakovac rivers together with the artificial lake Kruščica can also be present outside of the period of flooding.

During the dry period, the hygropetric in caves inhabited by *C. jalzici* visually resembles hygropetric in caves not subjected to flooding, as they are also characterized by slowly seeping and dripping water. But one key difference is that they are subjected to turbulent water movement during flood events that continuously wash these surfaces, and then, over the time of the water depletion, the suspended material is slowly depositing on the cave walls. Therefore, the results of this research confirmed that the hydrological conditions create a distinct habitat for the testate amoebae in caves that are subject to flooding. The impact of freshwaters on these habitats is also confirmed by the relatively high abundance of *Microchlamys patella*. It is an eurybiotic species, but is more dominant in the freshwater habitats (e.g. Yang et al. 2005; Vincke et al. 2006; Davidova and Vasilev 2012) in contrast to terrestrial and transitional habitats.

The influence of bat guano was also detected in results of Bray-Curtis similarity (Fig. 4), as the samples richest in bat guano (BUK_th, HOR_bg, SAM_sp) are assorted as separate branches, implying a specific response of testate amoebae on increased energy input and increased habitat heterogeneity (Mulec et al. 2016; Baković et al. 2022a). Specific influence of bat guano was also noted by Golemansky and Bonnet (1994), who mentioned a dense population of testate amoebae in bat guano deposits.

Conclusion

This study presents data on testate amoebae from eight caves of the Lika region in Croatia including the description of a new species for science, *Psammonobiotus dinarica* sp. nov. As heterotrophic protists in caves are scarcely researched, finding new species implies that caves could harbor more, still unknown, protists. The analyses from this study showed that testate amoebae assemblages differ in caves inhabited by *Congeria jalzici* in contrast to other caves studied. These differences can be attributed to the seasonal flooding that provides specific habitats for protists, as well as providing optimal conditions for the survival of endangered endemic *Congeria* species. This diversification needs to be further studied to better understand the conditions that shape specific protists assemblages. Except for the knowledge of cave testate amoebae, these cognitions could further enhance our understanding of the cave ecosystem and the conditions that sustain populations of *C. jalzici*.

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RESEARCH ARTICLE

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Evidence of ancestral nocturnality, locomotor clock regression, and cave zone-adjusted sleep duration modes in a cave beetle

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Abstract

The small carrion beetle *Ptomaphagus hirtus* is an abundant inhabitant of the exceptionally biodiverse Mammoth Cave system. Previous studies revealed negative phototaxis and the expression of biological clock genes in this microphthalmic cave beetle. Here we present results from probing *P. hirtus* for the entrainment of locomotor rhythms using the TriKinetics activity monitor setup. Although curtailed by low adjustment frequency of animals to the test environment, the data obtained from successfully monitoring two animals in constant darkness (DD) and six animals exposed to 12 hour light-dark cycles (LD) revealed a strong effect of light on locomotor activity in *P. hirtus*. In LD, activity was prevalent during the artificial night phases while close to absent during the presumptive day phases, suggesting conserved nocturnality. Upon transitioning LD animals to constant darkness, none displayed detectable evidence of free-running activity rhythms, suggesting complete regression of the central circadian clock. Equally notable, overall locomotor activity of the two DD-monitored animals was about three-fold lower compared to LD animals due to longer rest durations in the former. We, therefore, propose the existence of cave zone-specific energy expenditure modes that are mediated through light schedule responsive modification of sleep duration in *P. hirtus*.

Keywords

Introduction

One of the most fundamental discoveries in behavioral biology is the near-universal capacity of organisms to track circadian light level changes to the effect that the onset and termination of time-sensitive biological activities can occur in a stable, anticipatory manner, unmitigated by daily variation of light or temperature. Key evidence of this capability is the stunning continuation of behavioral rhythms in the absence of daily light level change as the zeitgeber, which has been documented in plants and animals alike (Bell-Pedersen et al. 2005). Pioneering studies in *Drosophila* defined the complex molecular machinery which facilitates this capacity in animals (Konopka and Benzer 1971; Patke et al. 2020). The critical significance of the biological clock for survival fitness and reproduction is documented by its regulatory sophistication, deep evolutionary conservation (Jindrich et al. 2017; Nikhil and Sharma 2017; Tarrant et al. 2019), and the large number of diverse fitness-reducing consequences of biological clock misregulation (Patke et al. 2020). At the same time, the question of exactly how the biological clock affects evolutionary fitness in natural populations continues to be a subject of ongoing research (Horn et al. 2019).

Cave-dwelling animals have long had a special place in biological clock research. This is for the intuitive prediction that adaptation to constant darkness should lead to the eventual evolutionary loss of the biological clock together with the well-documented dramatic loss of light perception-related traits such as eyes (Poulson and White 1969; Abhilash et al. 2017). Cave species are, if at all, only exposed to the diurnal changes of low-intensity light in the twilight zone areas of open caves (Beale and Whitmore 2016). Also temperatures stay relatively constant in this subterranean niche, usually fluctuating only by a few degrees Celsius within a year (Badino 2004). Thus, given the overall ecological constancy of cave environments, biological clocks seem dispensable in caves and destined to neutral evolutionary decay. And yet, studies designed to verify the "clocklessness" of cave species encountered a surprisingly widespread persistence of the biological clock in cave-dwelling organisms (Friedrich 2013; Beale et al. 2016). One of the best-studied examples is the Somalian cavefish *Phreatichthys andruzzii*. Although completely eyeless, *P. andruzzii* displays light avoidance by virtue of deep brain photoreceptor cells (Tarttelin et al. 2012). However, this residual light sensitivity notwithstanding, *P. andruzzii* is unreceptive to circadian activity entrainment by light despite the conservation and expression of an intact biological clock machinery gene repertoire (Cavallari et al. 2011). Regularly timed daily feeding, however, entrains anticipatory foraging behavior. Based on comparison with surface species, the behavioral entrainment by food provisioning represents an ancestral trait of *P. andruzzii* (López-Olmeda and Sánchez-Vázquez 2010), which remained conserved together with the biological clock gene complement. Equally remarkable, the molecular clock of *P. andruzzii* also supports the metabolic synchronization of cells, although at an expanded period of 30 hours (Cavallari et al. 2011). This example of what is known as "peripheral clocks", i.e. the autonomous activity of the biological clock machinery in a variety of tissues beside and independent of the circadian pacemaker neurons in the brain, is likewise well-documented in insects (Plautz et al. 1997).

Cave species like *P. andruzzii*, thus, raise the question of whether peripheral clock fitness benefits might continue to enforce conservation of the biological clock in cave environments (Lamprecht and Weber 1985; Vaze and Sharma 2013; Beale et al. 2016; Abhilash et al. 2017). In further support of this possibility, synchronized metabolic transcriptomes have been proposed to benefit the growth rates of fish larvae (Yúfera et al. 2017). Thus, while it is reasonable to predict that the circadian clock will regress in the absence of extrinsic advantages such as in the constant environments of caves (Shindey et al. 2017), the biological clock machinery itself may remain conserved due to its intrinsic advantages (Beale and Whitmore 2016). Cave species thus constitute an important resource to elucidate which functions of the biological clock are of intrinsic fitness significance besides optimizing the circadian behavior of surface species (Beale and Whitmore 2016).

In previous work (Friedrich et al. 2011), we performed an analysis of global gene expression in the adult head of the cave beetle species *Ptomaphagus hirtus*, an approximately 3 mm long small carrion beetle that is endemic to the highly biodiverse Mammoth Cave system in Kentucky (Packard 1888; Peck 1975; Culver and Hobbs 2017). This effort not only uncovered the expression of a large number of phototransduction genes but also of homologs of all insect core circadian clock genes, including *period* (*per*), *timeless* (*tim*), *Clock* (*Clk*), and *cycle* (*cyc*). Behavioral evidence of photophobic response to light produced further evidence of a highly reduced yet functional visual system, leading to the recategorization of *P. hirtus* as a microphthalmic cave beetle, in which the ancestral compound eyes have been reduced to relict eyelets with single lenses. Here we present the findings of efforts to probe for a functional circadian clock in *P. hirtus*, a scavenger, by testing for the light-entrainability of activity rhythms. While handicapped by a poor adjustment rate of laboratory-cultured animals to the experimental approach, our findings produced strong evidence that the circadian clock has regressed in *P. hirtus*, despite the central nervous system expression of biological clock genes. In addition, we present evidence of deep cave and twilight zone-attuned activity states in *P. hirtus* that may be the adaptive outcomes of nutrient abundance differences between the two ecological theaters.

Methods

Animal culture

P. hirtus adults were collected in March 2013 from White Cave entrance following guidelines defined in National Park Service permit MACA-2015-SCI-0019. Animals were cultured in a light-insulated cave laboratory room. Animals were housed in 60mm polystyrene Petri dishes supplied with a 50 millimeter deep bottom layer of cave soil. The Petri dishes were sealed off at the edges with parafilm. Culture dishes were placed in a Styrofoam box with a layer of moist paper towel at the bottom. The Styrofoam boxes were housed in an incubator at a temperature ranging between 10–12° degrees Celsius. Cultures were fed every two weeks with Fleischmann's Yeast pellets. All animal

handling was conducted under low-intensity red light, given the lack of light stress protective eye pigmentation in the eyelets of *P. hirtus* (Friedrich et al. 2011). Locomotor activity tests were conducted with offspring animals in 2017.

Monitoring of locomotor activity

Circadian activity was monitored using the Trikinetics Activity Monitor (TAM) (Trikinetics Inc., Waltman, MA, USA) placed in a Thermo Scientific Precision Low Temperature Incubator set at 11° degrees Celsius. Moist paper towel was secured to the bottom of the box to maintain humidity. Holes were poked in the plastic caps of the capillaries with a thumbtack for air circulation. Activity data were binned in 30-min intervals. For trials in constant darkness (DD), single beetles were placed into individual monitor capillaries and monitored for up to 14 days without the provision of food. For 12 hour light/12 hour dark regimen trials (LD), beetles were given a 5-hour acclimatization period in darkness, followed by the onset of 12h:12h light/dark cycles. RL5-W4575 White 75 Degree 4500 mcd LED lights (Super Bright LEDs Inc.) were used as light source powered at 2.5V and fixed 3 inches above the TAM, resulting in exposure of test animals to an approximate light intensity of 5×10^{16} photons/cm2/second during the 12 hour light phases.

Data analysis

Google spreadsheets and Actogram (Schmid et al. 2011) were used for the preparation of periodograms and actograms. The online implementation BoxPlotR (Spitzer et al. 2014) was used to generate summary box plots of activity intensities, activity periods, and rest periods. Nonparametric analyses of variance were conducted using the Kruskal Wallis Test Calculator hosted by Statistics Kingdom (2017): https://www.statskingdom.com/kruskal-wallis-calculator.html.

Results

Arrhythmic locomotor activity of *P. hirtus* in constant darkness

In addition to serving as the main tool in *Drosophila melanogaster* biological clock studies, the TAM setup has found increasing application for studying a broader range of insects (Bahrndorff et al. 2012; Giannoni-Guzmán et al. 2014; Pavan et al. 2016; Wang et al. 2021). Given the similar body sizes of *P. hirtus* and *D. melanogaster*, we explored the suitability of the original *Drosophila*-optimized TAM setup to monitor locomotor activity of adult *P. hirtus*, starting experiments in the light condition of deep cave zones, i.e. DD. Of a total of 28 individuals tested under these conditions in three separate trials, most perished within 2–5 days, after displaying high levels of locomotor activity (Suppl. material 1). Two individuals, however, completed an observation time span of 14 days alive (Fig. 1a, b and Suppl. material 2). Actogram inspection revealed
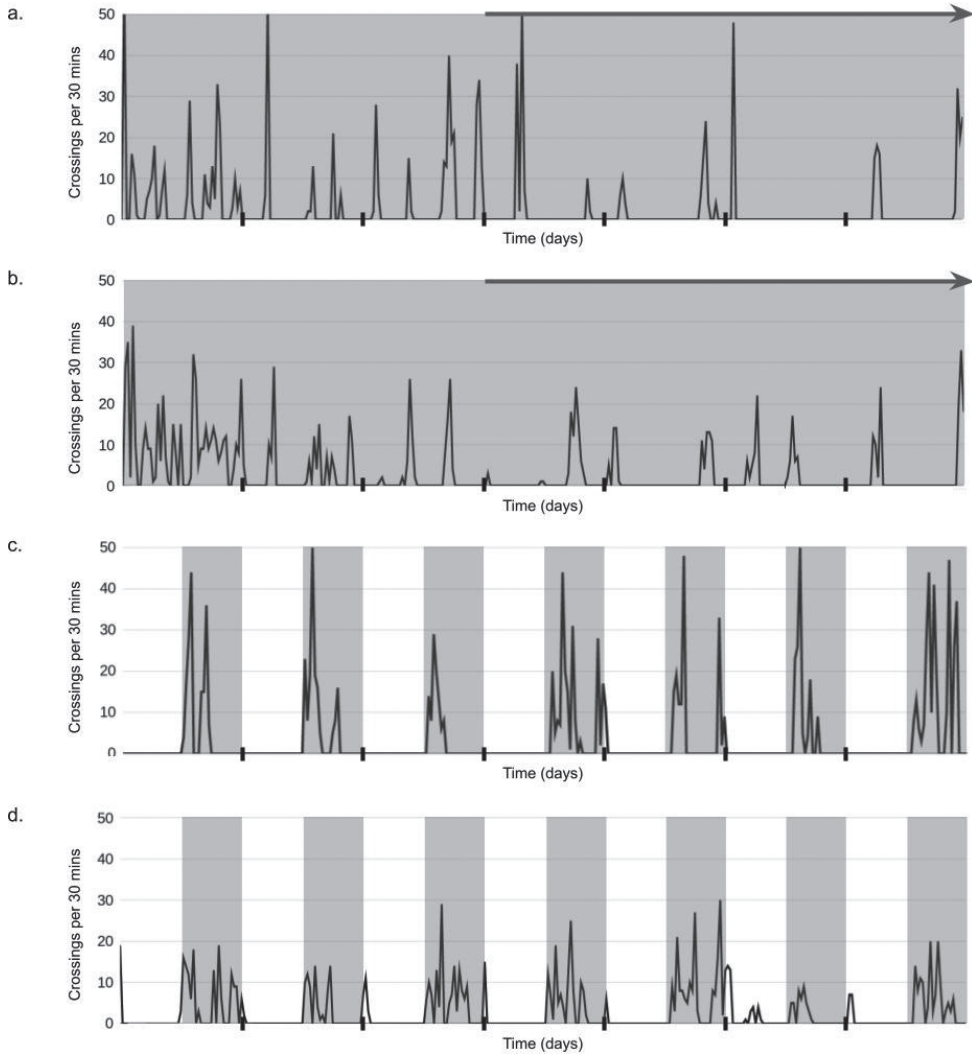
Figure 1. Initial 7-day activity profiles of *P. hirtus* adults under DD and LD. Graphs represent the first seven days of locomotor activity monitoring. Light-off phases indicated by grey overlays **a, b** adjusting animals in DD. Top right arrows indicate start of analyzed time span after the 3-day time window arbitrarily defined as adjustment phase **c, d** examples of adjusting animals in LD.

a decline of locomotor activity during the first three days of the trials. On day 1, individual locomotor activities averaged 29.4 and 18.2 crossings per hour, compared to an average of 14.5 and 11.2 crossings per hour on day 2, and followed by 5.2 and 3.3 crossings per hour on day 3. As the latter numbers were close to the averages of 4.3 and 8.3 crossings per hour observed on day 4, we concluded that the two animals had completed an initial phase of adjustment to the TAM capillary environment by the third day of monitoring. Activity patterns were therefore analyzed starting from day 4 of the observation period (Fig. 1a, b).

For the two adjusted animals monitored at DD, individual average locomotor activity levels amounted to 2.9 and 2.8 crossings per hour over 11.5 days of observation time following the adjustment phase. Further inspection of the corresponding temporal activity plots revealed that these low overall locomotor activities were the result of long resting phases interspersed by short activity bouts (Fig. 1a, b). The latter measured on average 1.6 (+/-0.8) and 1.3 (+/-0.7) hours for each animal in contrast to average resting phase durations of 9.8 (+/-6.5) and 13.8 (+/-10.4) hours. Peak resting phases exceeded 30 hours (Suppl. material 2).

Actogram analyses suggested a random distribution of activity bouts between resting periods (Suppl. material 3). Consistent with this, tests for periodicity in activity distribution failed to detect significant rhythmicity.

Presumptive night concentrated activity of *P. hirtus* exposed to 12h LD cycles

To test whether locomotor activity was affected by light in *P. hirtus*, we monitored animals exposed to 12:12 LD. Overall, the adjustment rate to the TAM environment was slightly higher compared to the tests conducted in DD, with six out of 28 animals tolerating the test tube environment for long-term, producing data for more than 20 days (Suppl. material 4). The activity profiles of adjusted LD animals differed conspicuously from that of the DD animals. Locomotor activity was concentrated in the artificial night (D) phases with an average of 15.6 tube crossings per hour (+/-3.3), which compared to only 1.3 (+/-0.7) in the L phases (Fig. 1c, d) Suppl. material 4). 80% of the L-phases were characterized by non-detectable locomotor activity (Suppl. material 4).

Higher locomotor activity in the LD-monitored animals

Given the activity pattern differences between DD- and LD-monitored animals, we asked whether the long-term LD-adjusted animals had expended more locomotor activity than the two animals monitored in constant darkness. Comparing the daily numbers of tube crossings revealed that the six LD-adjusted animals had performed an average of 191.3 (+/-36.3) tube crossings per day compared to 66.8 (+/-2.0) crossings by the two DD-adjusted animals (Suppl. materials 2, 4). These numbers revealed a close to threefold higher energy expenditure of the LD animals compared to the two DD-monitored animals.

Longer resting breaks of DD-monitored animals compared to 12h L/D-adjusted *P. hirtus*

The difference in daily activity amounts between the LD- and DD-adjusted animals could have been due to differences in activity intensities, activity phase durations, rest phase durations, or a combination of the three variables. To evaluate their relative impacts, we compared their ranges and averages between the LD- and DD-adjusted animals (Fig. 2). Given the preponderance of locomotor activity displayed by the LD-
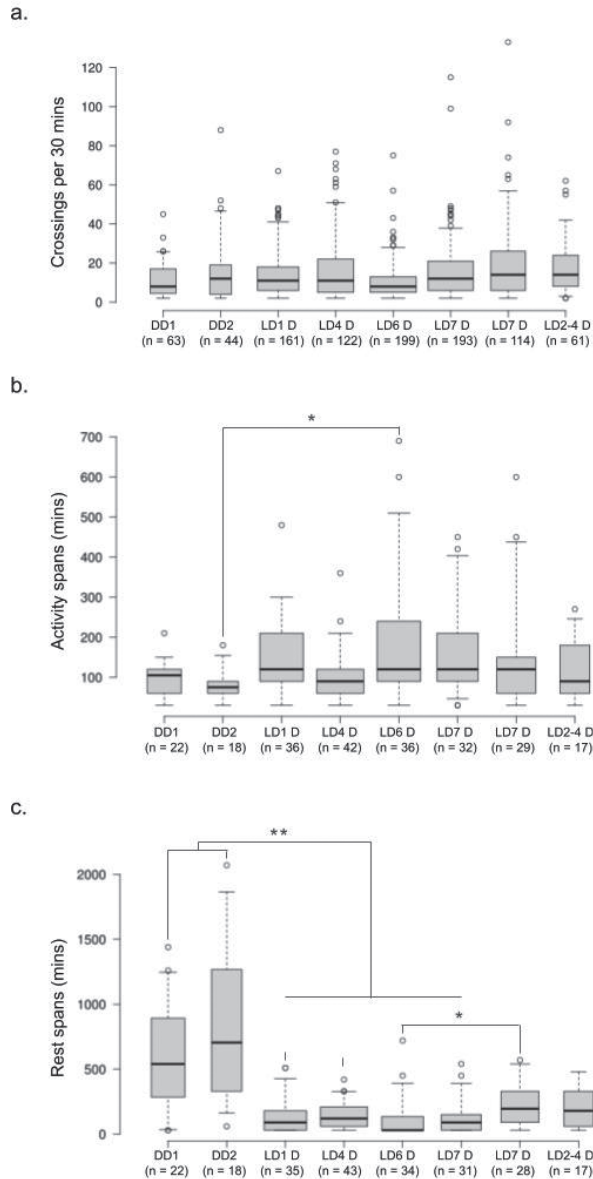
Figure 2. Analyses of rest-activity in long-term DD- and LD-adjusted animals **a–c** box plots, visualizing the relative numbers of observations as widths of boxes median values as back bars inside boxes. Altman whiskers extending from 5th to 95th percentile. Each comparison shows the results for the two long-term DD-adjusted animals (DD1 and DD2) followed by the results for the six long-term DD-adjusted animals (LD1, LD4, LD6, LD7, LD8, and LD2-4 in the D-phase **a** comparison of activity intensities measured as the number of tube crossings per discrete activity bout. Time intervals with less than 2 tube crossings were excluded. Numbers of bouts per animal is given in parentheses. Kruskal-Wallis ANOVA detected no statistically significant differences after adjustment for multiple comparisons **b** comparison of activity bout durations with numbers of bouts per animal given in parentheses **c** comparison of resting period durations with numbers of resting periods per animal given in parentheses.

adjusted animals in the presumptive night phases, we focused these comparisons on the D-phase activity profiles of the LD-adjusted animals.

The mean activity intensities of the two DD-adjusted animals were 11 and 16 tube crossings per half hour (Fig. 2a). These values fell within the range of mean D-phase activity intensities in the LD-adjusted animals, which spanned from 11 to 20 tube crossings per half hour (Fig. 2a and Suppl. material 5). None of the activity intensities were detected as significantly different from other tested animals.

The mean activity bout durations of the DD-adjusted animals were 94 (+/-50) min and 80 (+/-42) min (Fig. 2b). These numbers were closely below the lowest mean of 103 (+/-42) min in the LD-adjusted animals and close to twofold lower than the maximum mean of 164 (+/-110) min in the LD-adjusted animals (Fig. 2b and Suppl. material 5). There was also a conspicuous difference in the spread of maximum activity phase durations in the LD-adjusted animals, reaching up to 690 min in contrast to the maximum activity durations of 180 min and 210 min of the two DD-phase-adjusted animals (Fig. 2b, Suppl. material 5). These findings did indicate a possible contribution of activity bout duration to the net activity differences between LD- and DD-adjusted animals. However, only one DD-adjusted vs LD-adjusted animal comparison was detected as marginally significant (Fig. 2b).

The average resting period durations differed most prominently between the DD- and LD-adjusted animals. While the former were characterized by mean resting phase durations of 586 min and 828 min, mean resting phase lengths among the LD-adjusted animals ranged between 107 min to 231 min (Fig. 2c and Suppl. material 5). The maximum resting phase durations of 1,440 min and 2,070 min of the two DD-adjusted animals compared to 720 in the LD-adjusted animals (Fig. 2c, Suppl. material 5). Both DD-adjusted animals differed from four of the six LD-adjusted animals with high statistical significance (Fig. 2b). Taken together, these findings identified the variation in resting phase durations as the key variable underlying the net activity differences between LD- and DD-adjusted animals.

Lack of free-running activity rhythm

To probe whether the nocturnal locomotor activity rhythm of LD-cultured animals was entrainable in *P. hirtus*, we transitioned the LD-initiated animals into DD after 17 days. Without exception, all six animals discontinued their circadian activity rhythms, displaying stochastically occurring activity bouts (Fig. 3). Comparing the periodograms covering the DD period of seven days did not produce evidence of shared rhythms. The lack of evidence of free-running locomotor rhythms led us to conclude that the circadian locomotor clock has completely regressed in *P. hirtus*.

Discussion

The high lethality of tester animals in the TAM, as utilized in our experiments, confirms the systematic challenge of working with cave species (Mammola et al. 2021) and
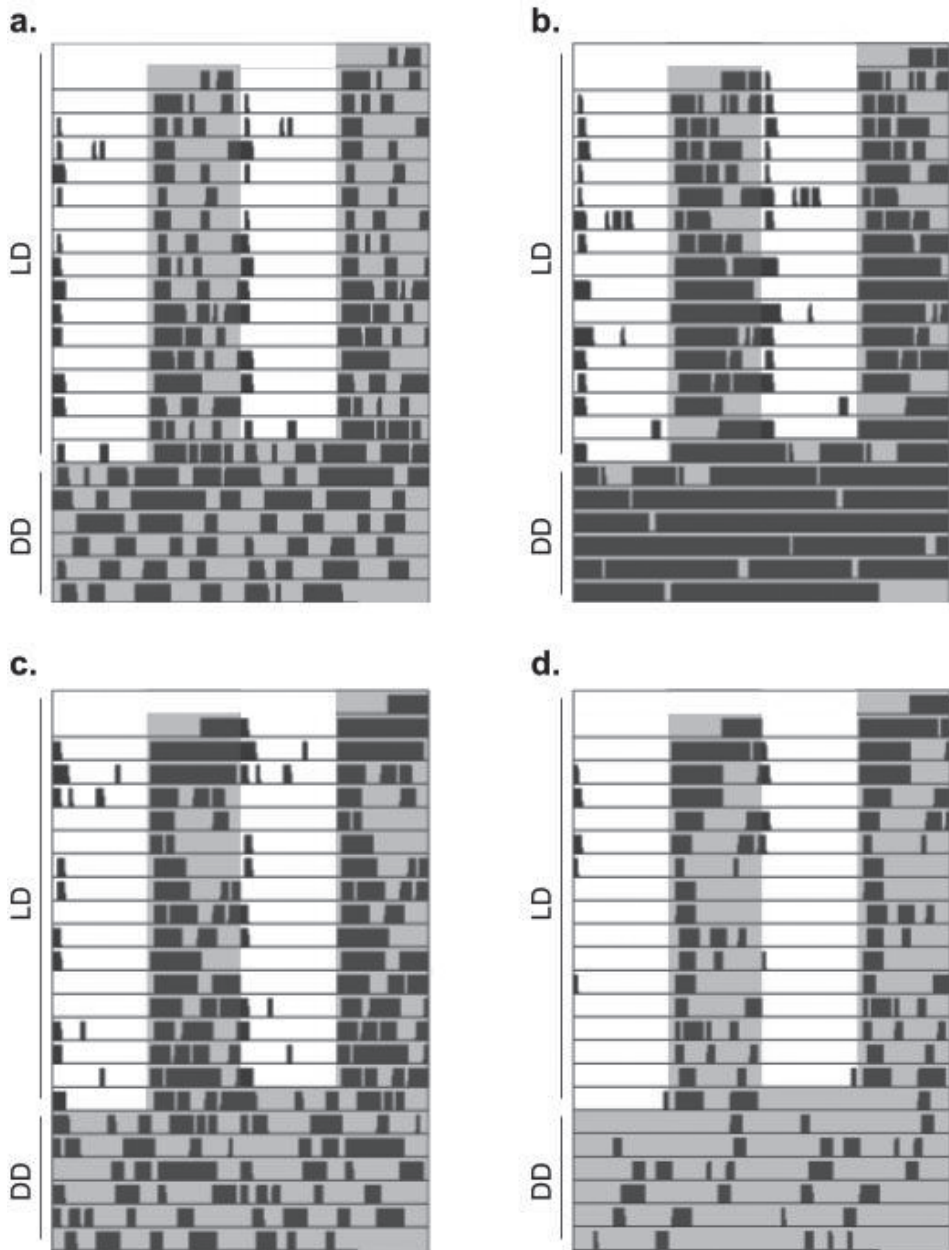
Figure 3. Lack of free-running circadian rhythm in LD-entrained animals. Actograms for LD animals 4, 6, 7, and 8 generated with Actogram (Schmid et al. 2011) at default settings. Each row represents two days, shifted by one day forward in relation to the previous row. Grey overlays indicate light-off periods.

reveals possible limitations of the TAM setup despite its well-documented versatility in surface species studies (Bahrndorff et al. 2012; Giannoni-Guzmán et al. 2014; Pavan et al. 2016; Wang et al. 2021). Future efforts will need to explore how food provisioning

and stringent humidity controls affect *P. hirtus* viability in the confinement of the TAM test tubes. In preliminary experiments, we find that *P. hirtus* adults can be cultured for over two months on moist filter paper on a weekly feeding schedule (MF, unpublished). Given the abundance and relatively easy accessibility of *P. hirtus* in the Mammoth Cave system, it should be possible to develop successful laboratory as well as field-based long-term monitoring approaches of *P. hirtus* locomotor activity.

Further evidence that *P. hirtus* is sensing light

While very limited in trial number and likely impacted by artificial stressors, our observations gained from monitoring two animals in DD and six animals in LD yield new compelling insights into the role of light in the biology of *P. hirtus* and provide preliminary evidence of possibly adaptive deep cave- vs twilight zone rest-activity (RA) modes in this microphthalmic cave beetle species.

    The first strongly supported finding of our study is that locomotor activity is affected by exposure to light in *P. hirtus*, given the highly nocturnal activity patterns of the six monitor-adjusted LD animals. This is further supported by the contrasting activity patterns of the two DD-adjusted animals. It is also consistent with the previously reported light avoidance of *P. hirtus* as well as the transcriptomic and structural evidence of a reduced but functional visual system (Friedrich et al. 2011). Given the likely lack of extraretinal light perception in *P. hirtus* based on the failure to detect homologs of extraretinal opsins in the available transcriptome data (Friedrich et al. 2011), it is most likely that the locomotor activity response to light is mediated through the *P. hirtus* eyelets.

Laboratory evidence that the locomotor clock has regressed in *P. hirtus*

As the second strongly supported finding of our study, our results leave little doubt that *P. hirtus* lacks the capacity to maintain a free-running circadian locomotor rhythm instructed by light as the zeitgeber. The previously reported expression of the biological clock gene network in the adult head may thus represent the activity of central pacemaker neurons responding to different zeitgeber sources. Alternatively, core clock gene expression may be associated with different outputs. In *Drosophila*, for instance, the expression of *Clk* and *cyc* in specific pacemaker neurons controls non-circadian rest-activity (RA) patterns and their maintenance throughout life history (Keene et al. 2010; Vaccaro et al. 2017).

    Transcriptome-based studies in samples of independently evolved cave populations of the Mexican cavefish *Astyanax mexicanus* revealed the parallel regression of both core clock gene regulation and target gene regulation (Mack et al. 2020), despite the conservation and non-retinal expression of a considerable number of opsin genes (Simon et al. 2019). Unlike in *P. hirtus*, however, locomotor rhythms are short-term entrainable (Espinasa and Jeffery 2006; Carlson and Gross 2018).

    Interestingly, cave populations of Asian loach (Family Balitoridae) species were found to display higher frequencies of conserved circadian clock penetrance and expressivity (Pati 2001; Duboué and Borowsky 2012). Together with the conserved

food provision-entrainable clock of *P. andruzzi* (Cavallari et al. 2011), these findings indicate different trajectories of central clock conservation in fish and beetles. This may be explained by the shorter time frame of cave colonization and the continued interbreeding with surface fish in the case of *A. mexicanus*. Further light on the generality of this difference could be gained by comparative studies of the considerable number of cave-dwelling congenerics of *P. hirtus* in the central and southeastern United States of America (Leray et al. 2019) and the large numbers of subterranean beetle species in the Palearctic and Australia (Leys et al. 2003; Ribera et al. 2010).

Conserved nocturnality: Ancestral adaptive response to predation pressure in the twilight zone?

Another strongly supported finding of our study is the D-phase activity preference of *P. hirtus*, when cultured in LD. This finding suggests that *P. hirtus* is a nocturnally active occupant of twilight zones in the open cave system of Mammoth Cave National Park. Our observations during collections confirmed the presence of *P. hirtus* in twilight areas, but we did not explore RA patterns in the field. It is interesting to note, however, that *P. hirtus* displayed a similarly strong level of photophobicity as its close relative *P. cavernicola* in light-dark choice tests (Friedrich et al. 2011). *P. cavernicola* is a facultative cave species, equipped with fully developed compound eyes (macrophthalmic) and flight-facilitating hind wings, and occupies a wide dispersal area in the Southeast of the United States (Peck 1984). While *P. cavernicola* has not yet been explicitly tested for nocturnality, this seems very likely given its strong avoidance of light. It seems reasonable, therefore, to speculate that nocturnality is an ancestral shared trait of *P. hirtus* and *P. cavernicola*. Ultimate evidence to probe this idea, however, will require comparative studies in both, closely-related cave- and surface species.

As the twilight zones of open caves constitute continuous links between surface and completely light-secluded deep cave space areas, it is further tempting to speculate that nocturnality originated in response to predation pressure at daylight. In further support of this, similar "nocturnal" activity profiles have been reported in all tested microphthalmic cave beetle species so far (Lamprecht and Weber 1977; Rusdea 1990; Friedrich 2013b). Also the phylogenetically more distant anophthalmic cave-dwelling amphipod *Niphargus puteanus* has been found to display a nocturnal activity profile when exposed to LD cycles (Gunzler 1965). Taken together, these data support the idea that nocturnality or crepuscular behavior are pre-adaptive traits for cave colonization (Romero 2011).

An obvious task towards gaining a better understanding of the significance of nocturnality in *P. hirtus* would be to elucidate the identity and biology of its assumed predators. Besides *P. hirtus*, Mammoth Cave is populated by six ground beetle species of the genera *Neaphaenops* and *Pseudoanophthalmus*, which are predators of small invertebrates, which might include larval or adult *P. hirtus* (Barr 1966, 1967; Culver and Hobbs 2017). Indeed, the most abundant of these, *Neaphaenops tellkampfii*, which is completely devoid of a visual system (Ghaffar et al. 1984) and highly specialized to prey on cave cricket eggs (Kane and Norton 1975), was found to consume *P. hirtus* larvae in laboratory culture

(Griffith 1990). In addition, it is possible that the massively abundant nymphal stages of the cave cricket *Hadenoecus subterraneus*, a likely omnivore (Levy 1976; Studier et al. 1986; Helf 2003), might exert daytime predation pressure on *P. hirtus* in twilight zones. *H. subterraneus* adults possess well-developed compound eyes, populate the cave ceilings, conduct nocturnal foraging dispersal into the surface environment, and have been reported to maintain a long-term free-running nocturnal activity rhythm (Reichle et al. 1965). The activity patterns of juvenile *H. subterraneus*, however, which populate cave floors reaching into the twilight zone at high frequencies, have not been explored yet. Last but not least, it is furthermore likely that surface species disperse into peripheral sections of the twilight zones, maintaining the ancestral predation pressure on nocturnality.

More resting in constant darkness: A cave-adaptive outcome of nutrient scarcity?

The final notable finding of our study is that LD-monitored *P. hirtus* displayed about three-fold higher locomotor activity than the two monitor-adjusted DD animals, mostly due to longer resting periods as opposed to differences in activity levels or activity bout durations. While preliminary, given the small sample size of our study, these findings raise the possibility that *P. hirtus* engages in light-contingent rest phase modes that may be adaptively optimized to cope with the contrasting nutrient provisions of the deep cave and twilight zone environments.

A rich body of studies in *Drosophila* confirmed that prolonged resting periods are generally reflective of physiologically and neurologically conserved sleep-like states in insects (Anafi et al. 2019; Beckwith and French 2019; Shafer and Keene 2021). Moreover, studies in vertebrate and invertebrate species converge on supporting the model that the amount of sleep and its circadian distribution are subject to regulatory mechanisms that are independent of the central clock (Borbély 1982; Borbély and Tobler 1996; Duboué et al. 2011; Duboué and Borowsky 2012). Of further potential relevance is that core biological clock genes regulate sleep frequency in *Drosophila*, as mentioned above (Keene et al. 2010). In light of these data from other species, it is reasonable to speculate that the RA patterns of *P. hirtus* are the outcome of conserved clock gene expression and yet independent of a central clock.

As a scavenger species, *P. hirtus* likely has to invest energy in foraging. The nutrient-poor environment of deep cave zones may enforce a more conservative energy investment strategy in the form of a lower food search frequency compared to the more nutrient-rich twilight zones. In the latter, *P. hirtus* likely enjoys higher probabilities of successful resource discovery, translating into a higher energy budget for sustaining foraging mobility and reproduction. It is therefore conceivable that *P. hirtus* may be characterized by cave zone ecology-adjusted sleep duration states.

Light regimen contingent sleep duration states have been reported in a number of cave-adapted fish species. In contrast to *P. hirtus*, however, cavefish species so far analyzed are characterized by higher amounts of energy expenditure in constant darkness due to the shortening of rest phases. This is true for the diverse cave populations of *A. mexicanus* as well as cave-adapted loach species (Duboué et al. 2011; Duboué and Borowsky 2012;

Carlson and Gross 2018). This difference between *P. hirtus* and cave-adapted fish may be due to different trophic structures of the aquatic subterranean environments of the cavefish populations and the terestrial deep cave zone populated by *P. hirtus*.

Drosophila has been found to respond to feeding scarcity with activity increase (Lee and Park 2004; Meunier et al. 2007; Keene et al. 2010; Yang et al. 2015). However, fruit flies can also be subjected to selection for starvation resistance, which results in activity reduction due to sleep phase extension (Masek et al. 2014; Slocumb et al. 2015; Miura and Takahashi 2019). It is, therefore, tempting to speculate that the proposed sleep phase extension state of *P. hirtus* in constant darkness may represent the result of long-term selection to tolerate food scarcity. Equally intriguing, suppressing flight capacity has been found to extend sleep duration in *Drosophila* (Melnattur et al. 2020). This raises the fascinating possibility that the adaptive sleep-extended state in the flightless *P. hirtus* may have originated as an integrated outcome of the complete regression of flight capacity. Taken together, our laboratory experiments with *P. hirtus* uncovered compelling but preliminary evidence of two new paradigms of cave adaptation, which invite further study in this and other cave species systems.

Conclusions

Given the small number of successfully TAM-monitored animals the findings of our study constitute preliminary evidence that the central locomotor clock has regressed in *P. hirtus*. Our findings further suggest that *P. hirtus* is nocturnally foraging in twilight areas of the Mammoth Cave system and may switch to longer resting periods in the deep cave areas to meet the challenge of more limited food resources.

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Supplementary material 1

Activity logs of select non-adjusting DD animals

Authors: Sonya Royzenblat, Jasmina Kulacic, Markus Friedrich

Data type: Trikinetics Activity Monitor output

Link: https://doi.org/10.3897/subtbiol.45.100717.suppl1

Supplementary material 2

Activity logs of the two long-term adjusted DD animals
Authors: Sonya Royzenblat, Jasmina Kulacic, Markus Friedrich
Data type: Trikinetics Activity Monitor output
Link: https://doi.org/10.3897/subtbiol.45.100717.suppl2

Supplementary material 3

Actograms of the long-term adjusted LD animals
Authors: Sonya Royzenblat, Jasmina Kulacic, Markus Friedrich
Data type: Trikinetics Activity Monitor output
Link: https://doi.org/10.3897/subtbiol.45.100717.suppl3

Supplementary material 4

Activity logs of the long-term adjusted LD animals
Authors: Sonya Royzenblat, Jasmina Kulacic, Markus Friedrich
Data type: Trikinetics Activity Monitor output
Link: https://doi.org/10.3897/subtbiol.45.100717.suppl4

Supplementary material 5

Activity stats comparisons of monitor-adjusted LD animals in D phase with monitor-adjusted DD animals

Authors: Sonya Royzenblat, Jasmina Kulacic, Markus Friedrich

Data type: Trikinetics Activity Monitor output

Link: https://doi.org/10.3897/subtbiol.45.100717.suppl5

RESEARCH ARTICLE

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A new cave population of *Astyanax mexicanus* from Northern Sierra de El Abra, Tamaulipas, Mexico

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Abstract

The *Astyanax* genus represents an extraordinary example of phenotypic evolution, being their most extreme examples the blind and depigmented morphs, which have evolved from independent surface-dwelling lineages. Among cave organisms, *Astyanax* cavefish is a prominent model system to study regressive evolution. Before this study, 34 cave populations were known for the *Astyanax* genus to be inhabited by the cave morph. The majority of those cave populations are distributed in Northeast México, at the Sierra Madre Oriental (32 cavefish), in three main areas: Sierra de Guatemala, Sierra de El Abra, and Micos, and two in the Balsas basin in the state of Guerrero, Mexico. In the present study, we describe a new cave population found 4.5 km Southward of Pachón cave, the most northern cave population known for the Sierra de El Abra limestone. El Refugio cave is a resurgence with a mixed population of fish with different levels of troglomorphism, and surface fish, resembling other hybrid populations within the Sierra de El Abra. Based on a mitochondrial DNA characterization of the 16S ribosomal DNA sequence, we could identify the mitochondrial lineage of this population, which was placed closely related to the "New Lineage", sharing haplotypes with the surface (i.e. Arroyo Lagartos) and Pachón populations, instead of with the

cave populations from Central Sierra de El Abra (e.g. Tinaja cave). El Refugio cave population gives additional evidence of the intricate history of this system, where migration, drift, and selection have shaped the evolution of the cave morphs through the independent episodes of the *Astyanax mexicanus* history.

Keywords

Astyanax, cavefish, regressive evolution, repeated evolution, Sierra de El Abra

Introduction

México represents a hotspot for troglobitic diversity in freshwater fish fauna, with seven different families with troglomorphic representatives including Characidae, Dinematichthyidae, Eleotridae Heptapteridae, Ictaluridae, Poeciliidae, and Synbranhidae families (Miller et al. 2005; Walsh and Chakrabarty 2016). This diversity of cavefish fauna is differentially distributed in the karstic regions of México, however, the Sierra Madre Oriental region, where cavefish populations of the *Astyanax* genus live (Characidae), includes a large number of cavefish populations, combined with a complex phylogeographic history, making this region a unique place to study regressive evolution.

Since the discovery of the first population of blind characins in 1936 in La Cueva Chica, in the southern portion of the Sierra del Abra limestone, by Salvador Coronado, the taxonomists of their time classified them as a new genus and species, *Anoptichthys jordani* (Hubbs & Innes, 1936), based on their troglobitic morphology (i.e., eye reduction and loss of body pigments). Shortly after, ichthyologists discovered another two cave populations of blind characids in the region. The first corresponds to El Pachón cave, *Anoptichthys antrobius* (Álvarez, 1946), and the second to *Anoptichthys hubbsi* (Álvarez, 1947), from Cueva de Los Sabinos, both at the Sierra de El Abra. At that time, each cave population was considered an independent episode of evolution from their surface ancestors and accordingly was described as different species. Afterward, Wilkens and Burns (1972), described the population of Micos, which was discovered in the 1960s, and characterized by fish that were not typically troglobitic, as they present similar pigmentation to surface fish, as well as a slight reduction in the vision. In terms of enzymatic diversity, they shown a clear differentiation from the previously studied troglobite populations (Avise and Selander 1972), and although Micos' diversity was not as high as in surface populations (being monomorphic for the majority of the loci), the authors shown that Micos cave presents the same alleles as the surface populations, suggesting that it is a recently adapted population, while others have suggested that it is a hybrid population (Mitchell et al. 1977). Thirty years later after their first discovery, during a multi-disciplinary exploration by Mitchell et al. (1977), cataloged 29 cave populations of *Astyanax mexicanus* (de Filippi, 1853) from Sierra de El Abra, Sierra de Guatemala, and Micos, in the states of San Luis Potosí and Tamaulipas in México. More recently, two new cavefish localities from the Sierra de El Abra, Chiquitita (Espinasa et al. 2018), and Toro #2 + La Calera system (Espinasa et al. 2020) were discovered. The recent discovery of new cave populations highlights the need to continue exploring this area, in order to better understand the extension and

connectivity that exists within the hydrological systems of the Sierra de El Abra, Sierra de Guatemala, and Micos areas.

Due to the extreme differences between the surface and cave *Astyanax mexicanus* morphs, the cave-dwelling morph has been considered a model organism to explore the genetic mechanisms underlying regressive evolution, particularly, vision loss, as well as the reduction in pigmentation (Protas et al. 2007; Gross et al. 2008; Protas et al. 2008; McGaugh et al. 2014; Jeffery et al. 2016). In recent years, the unprecedented development of molecular biology, developmental biology, and genomics, has allowed researchers to scan at the genome level the evolution of cave-adapted morphs in the Mexican tetra *Astyanax mexicanus*, not only in their phenotype, but also in their physiology, metabolisms, and behavior (McGaugh et al. 2014; Riddle et al. 2018; Ma et al. 2021; O'Gorman et al. 2021).

Regarding the evolutionary history of the cavefish populations of *Astyanax mexicanus*, multiple studies have shown evidence that cave populations have originated from two independent lineages (Strecker et al. 2004; Ornelas-García et al. 2008; Bradic et al. 2013; Herman et al. 2018), giving rise to parallel evolution of cave morphs within the species. In this regard, the cave populations from Sierra de El Abra area have been recognized as a different cave lineage, previously known as the old lineage, from the Micos and Sierra de Guatemala, previously known as the new lineage (Wilkes 1988; Gross 2012). Considering the hydrogeology of the region, five major drainages have been suggested (Espinasa and Espinasa 2016; Elliot 2018; Espinasa et al. 2020): 1) Sierra de Guatemala, draining into the Florida, Río Frío and Río Nacimiento springs, 2) Northern Sierra de El Abra, draining into Río Mante, 3) Los Sabinos in Sierra de El Abra, draining mostly into Río Choy, 4) Southern Sierra de El Abra, draining mainly to the Tampaon river, and 5) Micos, draining into Valles River.

With the development of genetic tools, it has been possible to characterize the evolutionary history and phylogeographic patterns of cave populations in northeastern Mexico. Among the first studies carried out with the mitochondrial DNA data, *Astyanax mexicanus* cavefish showed the presence of two different lineages that were named by Dowling et al. 2002 as lineage A and B (also known as the new and old lineages, see Gross 2012). The lineage A included the Northern and Southern extremes of the Sierra de El Abra (i.e. Pachón and Chica caves), together with the Sierra de Guatemala and Micos caves, grouped with most of the local surface populations. While the caves with the haplotype B included the Yerbaniz and Los Sabinos area *sensu* Elliott (2016) in the central Sierra de El Abra, (Dowling et al. 2002; Strecker et al. 2004; Ornelas-García et al. 2008; Bradic et al. 2012; Espinasa et al. 2020). Based on genomic data, we now know that the extremes of the Sierra de El Abra (i.e. Pachón and Chica) belong to the same lineage that the rest of the Sierra de El Abra caves, but they have undergone a mitochondrial introgression with surface fish (Bradic et al. 2013; Herman et al. 2018; Moran et al. 2022). Moreover, the Sierra de El Abra corresponds to an independent lineage to the Sierra de Guatemala and Micos corresponding to independent events of cave adaptation (Bradic et al. 2012; Strecker et al. 2012; Herman et al. 2018). In addition, we know that in some populations from the Sierra de El Abra, Micos, and Sierra de Guatemala, there has been gene flow with surface populations and this contact has been differential between the populations of these areas (Bradic et al. 2012; Herman et al. 2018; Moran et al. 2022). Thus, the complete interfertility between surface

and cave populations has allowed the exploration of the genetic architecture underlying phenotypic changes in the cave-adapted morphs (Hubbs and Innes 1936; Alvarez 1946, 1947; Sadoglu 1956; Wilkens and Hüppop 1986; Wilkens 1988). Finally, based on coalescence methods, and important genomic representation, it has been possible to support the hypothesis proposed by Avise and Selander (1972), that the origin of cave populations is very recent (<200,000 years, Fumey et al. 2018; Herman et al. 2018), contrary to what had been suggested with mitochondrial molecular clocks (Strecker et al. 2004; Ornelas-García et al. 2008).

In the present study, a new population is described for the northern region of Sierra del Abra. A general description of its geohydrology is included. In addition, a brief description of the phenotypic variation found in the cave, and the characterization of a mitochondrial ribosomal marker (r16S) were included. Based on this information, it is concluded that this population could correspond to a mixed population, with a large phenotypic diversity in terms of the degree of regression of vision and pigmentation traits, as has been reported in other hybrid caves such as Chica. Regarding the geometric morphometric variation, we recovered El Refugio populations as intermediate between the Pachón and Tinaja caves, giving evidence about the common ancestry between the caves within the Sierra de El Abra system, despite the large introgression between the Río Lagartos surface population and El Refugio cave.

Methods

The "El Refugio" cave (22°34'28.50"N, 99°01'30.74"W, 231 masl), takes its name from the closest town in the state of Tamaulipas, México. The cave was discovered by Jean Louis Lacaille in April 2020, and troglomorphic fish were seen in some of the cave pools (Figs 1, 2). A second expedition trip was organized on February 23th, 2022 with the purpose of fully documenting and collecting specimens of this cave. On that day, a spring only 100 m from the Refugio cave (22°34'25.50"N, 99°01'29.61"W) was also visited. The Manuel de la Fuente spring is a 3 m deep open-air well from which the locals extract water for consumption using a pipe (Fig. 3). The well harbors an electric pump. Upon inspection, two cavefish were seen swimming under the sunlight. A cable ladder was used to descend the well and collect both fish with a hand net. Underwater conduits were evident at the bottom of the well from which fish could swim out, but they are probably too small for human exploration with scuba equipment.

To explore the El Refugio cave, a cable ladder was also employed to access its 2 m entrance pit. The cave was surveyed using a DistoX2 device, and the TopoDroid app on a tablet. Survey data were processed using Visual Topo ver. 5.17, and drawn using Adobe Illustrator. To capture specimens, hand-held nets were used. Photographs were taken in the field with the help of a small glass tank and an iPhone 11 camera. A small fin clip was taken with scissors for DNA studies from all specimens. Voucher samples were stored at the Colección Nacional de Peces, IBUNAM, Mexico.

Figure 1. a simplified map edited from Elliott (2016), with the new cavefish locality of El Refugio, as well as the localities included in the morphological characterization: Pachón and Tinaja in Sierra de El Abra system **b** position of the 12 homologous landmarks (red) and the 15 pseudolandmarks (blue) curve for geometric morphometric analysis. Within the eye orbit the vertical line (red line), corresponds to the Orbital Diameter Height, and the horizontal line (blue line) corresponds to the Orbital Diameter Length.

Figure 2. Highly troglomorphic fish are found in the El Refugio cave and in the nearby well. The two fish in the insert were found in the well-used by locals to extract water. In this well, troglomorphic fish are seen swimming under the sunlight. The most troglomorphic fish in this cave system have two types of colorations, pink (**A**) and white (**B**). These two colorations are shown also in the specimens in the insert. Notice also that eye regression and skull modification create a downward hump in some specimens (red arrow).

For the collection of cave specimens, permission was obtained from the competent Mexican authorities (SEMARNAT SGPA/DGVS/03334/22). All of 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 samples were obtained following standard methods for DNA purification using Qiagen's DNeasy Tissue Kit by digesting a fin clip of the individual in the lysis buffer. A fragment of the mitochondrial 16S rRNA was sequenced for 6 troglomorphic, one hybrid, and one epigean fish, all collected inside the El Refugio cave. The 16S rRNA fragment was amplified and sequenced using the 16Sar (CGCCTGTTTATCAAAAACAT) and 16Sb (CTCCGGTTTGAACTCAGATCA) primer pair for 16S rRNA. Amplification was carried out in a 50 μl volume reaction, with QIAGEN Multiplex PCR Kit. The PCR program consisted of an initial denaturing step at 94 °C for 60 sec, 35 amplification cycles (94 °C for 15 sec, 49 °C for 15 sec, 72 °C for 15 sec), and a final extension step at 72 °C for 6 min in a GeneAmp PCR
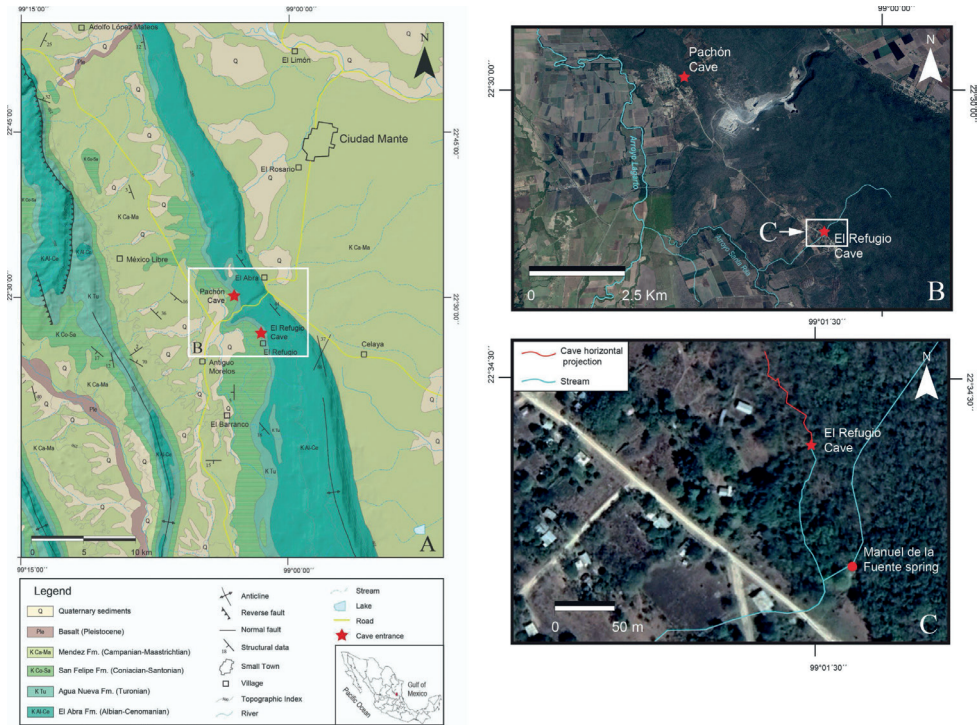
Figure 3. A general geologic map of the northern part of the Sierra del Abra showing the locations of the El Refugio and Pachón caves entrances at the geological contact between Agua Nueva and San Felipe formations (modified from Carta Geológico-Minera Ciudad Mante F14–5) **B** satellite view showing the principal streams near the cave entrances **C** detailed view of B. The cave horizontal projection of the El Refugio cave is observed in a red line as also the position of the Manuel de la Fuente Spring. In blue are stream beds that only carry water during the rainy season.

System 9700 (Perkin Elmer). PCR amplified samples were purified with the QIAquick PCR purification kit and directly sequenced by Elim Biopharm Genomic Services (https://www.elimbio.com/services/dna-sequencing/). Chromatograms obtained from the automated sequencer were read and contigs were made using the sequence editing software Sequencher™ 3.0. External primers were excluded from the analyses. BLAST was used to identify GenBank sequences that resemble the specimens. Sequences were aligned with ClustalW2.

For comparison, DNA sequences were aligned to sequences from localities reported previously by Espinasa et al. (2020). We included the mitochondrial haplotypes classified as lineage A or B, which for lineage A the following populations are included: Comandante surface river (affluent of Río Frío), Sótano del Molino, Sótano del Caballo Moro, Cueva del Pachón, Toro #2, Cueva Chica, and Cueva Chiquitita. Mitochondrial lineage B included samples from the Rascón surface stream, Tamasopo surface stream, Cueva de Los Sabinos, Cueva de la Tinaja, and Cueva de la Curva.

Morphological characterization

To characterize the morphospace of the El Refugio population and their relationship with other populations from the Sierra de El Abra, we performed landmark-based Geometric Morphometrics (GM) analysis on the lateral left-hand view of the body shape. We digitized 12 homologous landmarks and a curve with 15 pseudo landmarks with the TPSDig2 v. 2.31 (Rohlf 2015; Fig. 1), of 45 organisms, 19 from El Regio (8 completely troglomorphic, and 1 apparently from the surface, and 10 with intermediate characteristics), 10 from Pachón, and 10 from Tinaja, and 6 from a surface population (from Río Florido, Pánuco basin). A Generalized Procrustes Analysis (GPA) was performed with the "gpagen" in Geomorph v. 4.02 (R Core Team 2019), and with the 'curve' argument the sliders were defined with the Procrustes distance criterion to optimize the position of the sliding reference point during the GPA.

To avoid a size effect by allometry, the residuals of the regression of the shape on the centroid size (CS) were calculated with the function "procD.lm". Once the residuals were obtained, an allometry-free shape was created, using these residuals of the morphological data. The analyses were made on this allometry-free shape. To assess the variation in body shape, we used principal component analysis using the "gm.prcomp" function. This is an ordering analysis that captures the multidimensional variation that is inherent in body shape (Zelditch et al. 2004). Deformation grids were used to describe the morphospace at the ends of the axes, which allowed us to observe the difference in the relative shape associated with the components that explain a greater percentage of variance (Zelditch et al. 2004).

In addition, discriminant analysis of principal components (DACP) (Jombart et al. 2010; Jombart et al. 2011) was performed in RStudio (RStudio, Team 2020). DAPC is a multivariate analysis designed to identify and describe clusters of morphologically related individuals. DAPC relies on data transformation using PCA as a prior step to Discriminant Analysis (DA), ensuring that variables submitted to DA are uncorrelated. DA method defines a model in which genetic variation is partitioned into a between-group and a within-group component and yields synthetic variables that maximize the first while minimizing the second (Jombart et al. 2010). DAPC was performed using the geometric morphometric data comparing the individual assignment considering the "find.clusters" function. We run a K-means clustering algorithm (which relies on the same model as DA) with different numbers of clusters, each of which gives rise to a statistical model and an associated likelihood. With the "find.clusters" function, we evaluate from K = 1 to K = 6 possible clustering in 10 different iterations (DAPC) (Jombart et al. 2011). The selection of the number of principal components was carried out with a cross-validation analysis. The clusters or conglomerates resulting from the DAPC were visualized in a scatter diagram, using the first two discriminant functions, representing individuals as points. A variance analysis was carried out, using the population as a factor (i.e., El Refugio, Pachón, and Tinaja), and the Centroid size as a covariable, as well as the interaction between population and CS, was estimated in R ver. 3.6.1 (R Core Team 2019).

An Orbital Diameter Height was estimated (red line in the orbit, Fig. 1b), and the Orbital Diameter Length (blue line in the orbit, Fig. 1b), both measures were corrected by the standard length of the fish, and a boxplot was carried out to graphically compare the median and standard deviation among Pachón, El Refugio and Tinaja cave populations, in contrast with a surface population (i.e., Río Florido).

Results

El Refugio cave is part of a series of resurgences (i.e., point at which an underground stream reaches the surface and becomes a surface stream) in the area that include the nearby well and Refugio cave itself. The cave shows tectonic control, and the orientation of the galleries follows the main NW-SE fault (Fig. 3A). Cavefish were encountered at two close localities: 1) El Refugio cave (Fig. 3B), and 2) at the Manuel de la Fuente spring (Fig. 3C). The two localities are aligned to the fault direction and separated only 100 m from each other, suggesting that they are hydrologically connected and allowing the cavefish to move from one to the other.

Regarding surface drainage, during the dry season water is pumped out through the well, but during the rainy season, high water volumes flow from both localities. During the rainy season, water from the Manuel de la Fuente spring and El Refugio cave resurgence join forming a creek that flows to the Arroyo Santa Rita downstream and is captured by Arroyo Lagartos (Fig. 3B, C).

Cave description

The El Refugio cave has a 2 m pit entrance that leads to a single gallery that develops parallel to a local fault going northwest with an average direction of 330°. The cave's total length is 98 m and has a 4 m total depth. The cave ceilings are low, and therefore requires crawling most of the time. El Refugio is a shallow cave that acts as a resurgence in the wet season, where water floods the entire gallery and flows out from the cave entrance. El Refugio cave has a few small pools the cave forms of *Astyanax mexicanus* as well as surface and intermediate fishes (Fig. 4).

During the dry season when El Refugio cave was explored, dry galleries were interspersed with some pools. The first was a very shallow puddle under a small bat colony (*Astyanax* pool 1 in Fig. 4). Only a surface fish and a troglomorphic fish were spotted. The second pool is a crevasse about 1 m long (Fig. 5A), but several meters deep, hosting tens of fish. A deep crevasse appears to join further underwater passages (Fig. 5B). Many different cavefish were seen going into and out of this void, suggesting a large underwater conduit inaccessible to humans, perhaps joining the aquifer (*Astyanax* pool 2 in Fig. 4). The third pool was the longest, about 10 m long, and was inhabited by both *Astyanax mexicanus* cavefish and mysid cave shrimp *Spelaeomysis quinterensis*. Low ceilings make you crawl inside the water, although an air current hints that the cave has furthermore unexplored passages (*Astyanax* pool 3 in Fig. 4).
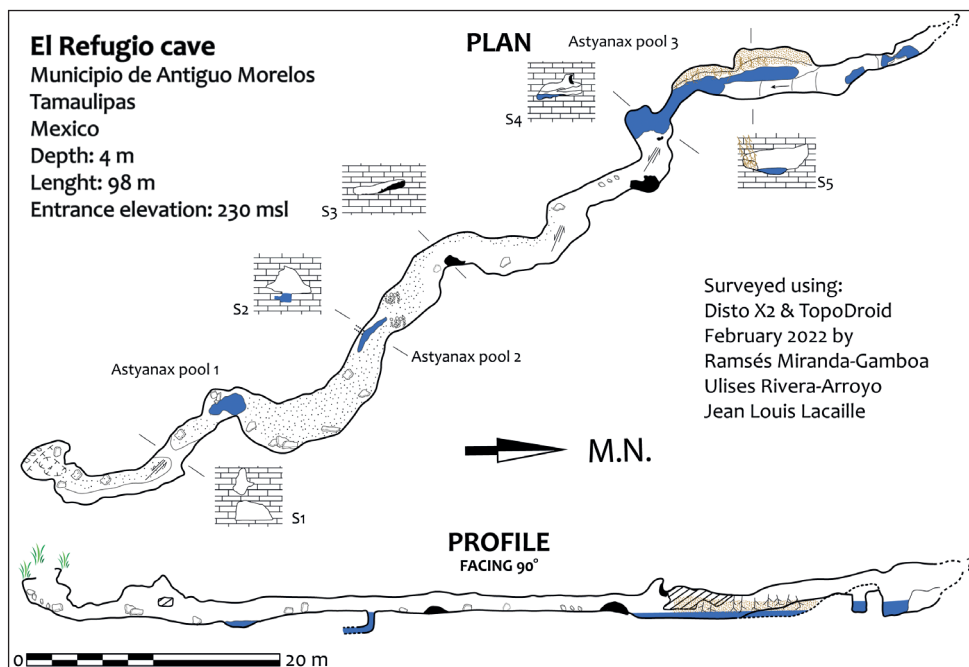
Figure 4. Map of El Refugio cave.

The initial part of the cave has a bed of sediments and guano from hematophagous bats that shelter in the cave. On the left wall of the third pool, there is a long mound of mud attached to roots coming from the ceiling or the walls, indicating a near connection to the surface (Fig. 4). In the final part, there is a little slope reaching a couple of deeper pools without cavefish. In the second one an apnea is required to get to the last chamber. In this part, the ceiling is high enough to stand straight. The cave ends with a small crack impenetrable for humans but seems several meters long (Fig. 4, dashed line at the farthest part of the cave in the profile and plan).

El Refugio is an active cave that discharges water in the rainy season and as such, inside the cave, there is evidence of this. All the Refugio walls are covered with scallops showing the dynamic behavior of water (Fig. 5B). There are plenty of dissolution domes in the ceiling. They have different sizes, most of them are about 15 cm long, as can be seen in Fig. 5C, but a couple of them are about meter-sized (Figs 4, 5D) and are located principally just over fissures allowing the possibility of water to seep up and dissolve the rock.

As it is possible to observe in the cross sections of the El Refugio cave, has the characteristic shape of a phreatic conduct whose shape is elliptical (Fig. 4), and develops along the fissure axis as can be seen in Fig. 5E, F. This suggests a slow flow of floodwaters through the cave. Furthermore, there is another evidence of what possibly is a local water table. Many walls of the cave have calcite adhered to them, as is shown
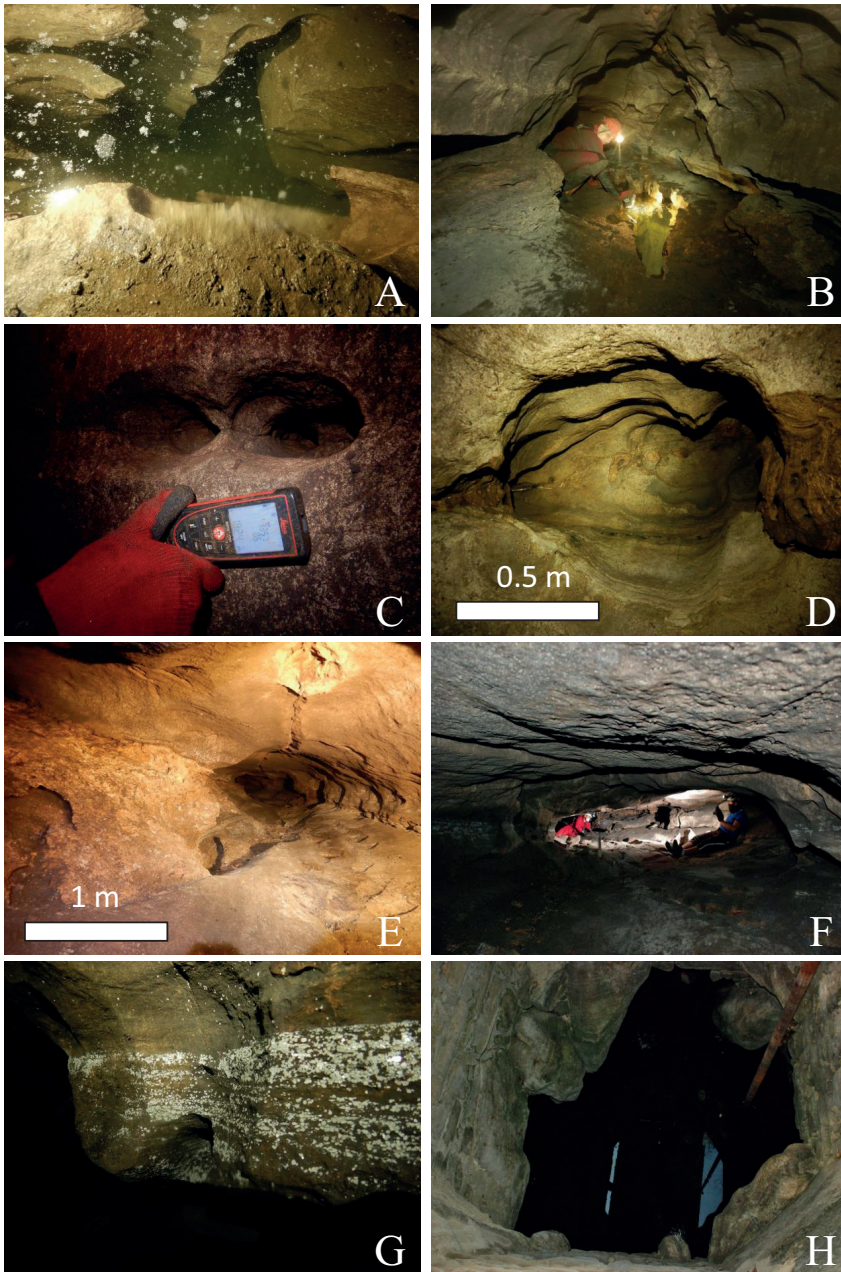
Figure 5. Some important features of the cave **A** an underwater crevasse about 1 m long in the second pool **B** aforementioned crevasse on the floor leading to underwater passages **C, D** dissolution domes of different sizes along the cave galleries, suggesting that during rainy season, the whole cave is under water **E, F** examples of phreatic conducts with a typical elliptic morphology developed along fissures **G** calcite crystals covering the walls cave as evidence of multiple variations in the water table **H** view from the surface of the Manuel de la Fuente well.

in Fig. 5G, indicating that the water level remained in the same position for a long time. Additionally, there is a testimony of local people who actually saw in May of 2021 that the water level was higher than the one documented in February of 2022. Finally, the well (Fig. 5H) which also hosts blind fishes and probably is connected to the El Refugio cave, is a year-round source of drinking water for the people of the El Refugio community and its level doesn't decrease over time. All these are evidence that El Refugio cave is related to a water table or aquifer.

Specimens

This cave hosts a mixed population; thus, we can find troglobitic (with complete loss of vision and pigment), surface and intermediate morphs. From the cave survey of the specimens that were observed in detail, 31.8% (N = 7) were highly troglomorphic specimens, with no pigment or external remnants of eyes, 54.5% (N = 12) were intermediate with either some type of eye and/or pigment, and 13.6% (N = 3) were surface-like fish. It is likely that surface fish are more abundant within the population, but since they react to light, they are more difficult to catch and swam away. The troglomorphic fish had either characteristic pinkish or white colorations (Fig. 2). The surface-like fish were fully pigmented, with large eyes, and as mentioned, responsive to light. It is the authors' assumption that they are simply surface fish that during the rainy season swam inside. While during the dry season there is not a surface stream, it is expected that when flowing in the rainy season, the temporal stream gives easy access to surface fish from the nearby Arroyo el Lagarto. Introgression between the surface morph and the cave morph is suggested by the presence of individuals with a large phenotypic variation in the eye-regression level, from entirely absent eyes, to embedded eye cist, embedded small eye, a small eye with closed pupil, a small eye with open pupil, to large eyes (Fig. 6A–F). Similarly, we could observe a wide variation with respect to the combination between body pigments and eye regression levels, that is small-eye or eyelss without pigment, eyed and eyeless with a yellow pigment, eyeless with a back caudal spot and eyed fish with black spot and yellow pigments at the caudal fin (Fig. 6G–L).

Mitochondrial DNA

A 16S rRNA fragment of 572 bp was sequenced for six troglomorphic fish, one hybrid, and one epigean fish from El Refugio cave. All specimens analyzed have the haplotype A (GenBank# AP011982.1), identical to the Pachón cave sequence and to the local surface population (i.e., Arroyo Lagartos). When comparing El Refugio sequences to the mitochondrial haplotype B from Rascón and Tamasopo surface streams fish, El Refugio specimens differed by 2–3 bp. When we compared El Refugio sequences with Sabinos, Tinaja, and Curva caves sequences, they differed by 5 bp. Thus, our 16S rRNA sequences recovered El Refugio cave specimens closer to the northern Sierra de El Abra region, instead of the Central part of the Sierra de El Abra (Suppl. material 1).
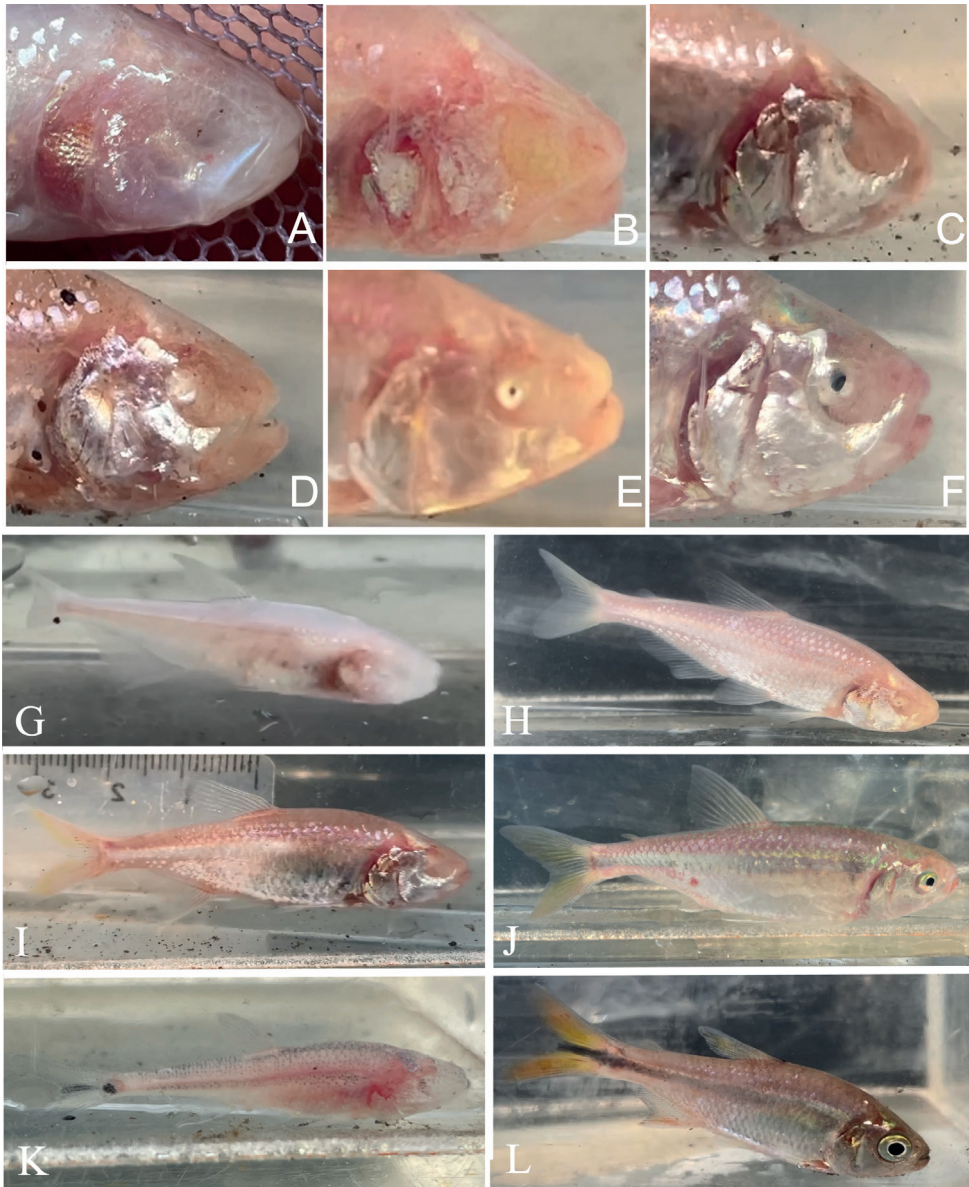
Figure 6. Eye regression variations within the El Refugio population. In the figure, we can observe from entirely absent eyes (**A–C**), to different levels of vision regression (**D–F**). Regarding the body and eye regression combined we also observed a wide variation **G, H** eyeless and small-eye fish without pigment **I, J** eyeless and eyed fish with yellow pigment, and eyeless fish with a caudal black spot (**K**) **L** eyed and black spot and yellow pigment in the caudal fin.

Morphological variation

The first two components explain 43.67% of the cumulative variance (Fig. 7A, Suppl. material 2). PC1, which explains 25.84% of the variance, describes the variation in
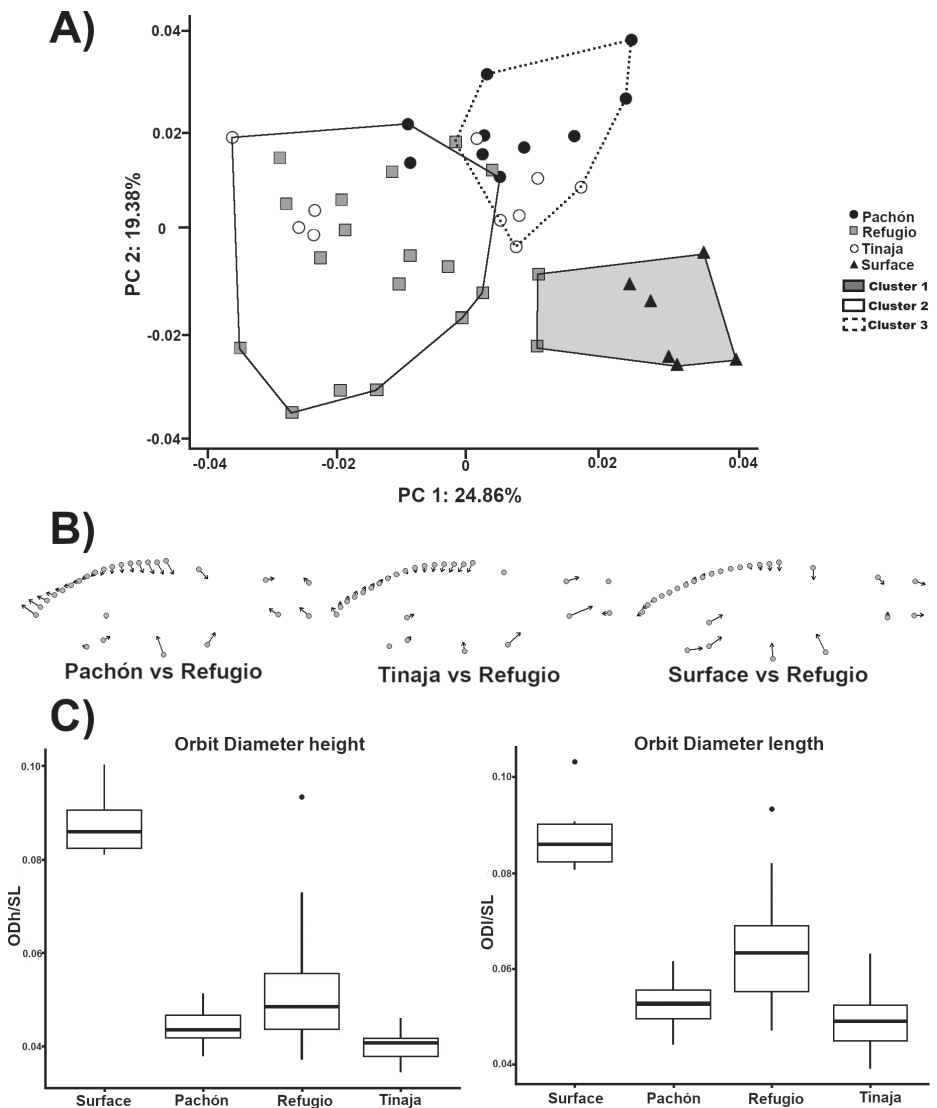
Figure 7. A PCA of the body shape of the three cavefish populations. At the end of each axis, the shape obtained by the positive and negative ends was placed. The hullboxes delimit the clusters obtained through K-means analysis. Black hullbox = cluster 1 (surface morph), Filled hullbox = cluster 2 (intermediate individuals), dotted line hullbox = cluster 3 (the most troglomorphic individuals) **B** comparison of the consensus shape of the El Refugio population (deformation vectors) vs. the consensus shapes of Pachón, Tinaja, and Surface (points) **C** boxplot of the orbital diameter corrected by the SL for the Surface, Pachón, El Refugio and Tinaja populations.

body height, with slender bodies at the negative side of this component, and deeper bodies at the positive side. The PC2 explains 17.83% of the variance, and we observed differences in the dorsal profile of the head, discriminating between troglobitic and surface individuals, thus, at the most positive side of PC2 we recovered the Pachón, Tinaja

and El Refugio individuals with a concave head profile (i.e., duck-bill-like rostral flat-tening), and at the negative side of the PC2, we recovered the individuals with a softer and streamlined dorsal profile of the head, characteristic in surface-like fish (Fig. 7A).

For the DAPC, a total of 38 PC were used, and based on the Silhouette method we recovered K = 3 as the optimal number of clusters (Fig. 7A). According to this result, the first cluster (black hullbox) gathered the surface population (i.e., Río Florido), with two surface-like individuals from El Refugio. The second cluster (filled hullbox) with the largest morpho-space considering both PC axes, grouped most of the fish from El Refugio population, with intermediate morphs between the surface and the cave individuals. Although in this cluster we find Pachón and Tinaja fish, both are distributed on the positive side of PC2, with a concave dorsal profile of the head, while the El Refugio fish specimens were characterized by a slender body, with a broad distribution in the second axis (PC2), corresponding to a gradient in the degree of dorsal flattening of the head. Finally, we have the third cluster (dotted line hullbox), which included fish from Pachón, Tinaja and two individuals from El Refugio, which correspond to the most troglobitic fish.

The MANCOVA support differences among populations, but not by the centroid size or the interaction between CS and the populations (Table 1). These differences among populations can be appreciated in the comparisons among the consensus forms obtained for each population (Fig. 7B). In both comparisons, we use the Pachón and Tinaja cave populations as the reference (the gray points), while El Refugio was depicted by the deformation vectors. In general, the differences for El Refugio population were similar with respect to the other two populations, thus, it has a slender body in contrast to the other two cave populations, and additionally, Pachón cave presents a stronger duck-bill-like rostral flattening. In Tinaja the caudal peduncle was larger than the other two populations (Fig. 7B).

Finally, in the orbital diameter bloxplot (Fig. 7C, Suppl. material 2), was possible to observe a wide variation in the El Refugio population, corresponding to a wide spectrum of phenotypes, from the most troglomorphic with a completely absent eye to the individuals with a surface-like eye, giving additional evidence of the mixed nature of this population. In Table 2 we present the average values for the orbital diameter measurements and their proportions with respect to the standard and the head length. For El Refugio population, the average orbital diameter height (OD_H) is 2.35 mm (SD = 0.77 mm), and the average orbital diameter length (OD_L) is 2.88 mm (SD = 0.72 mm). While the average values were lower in both Pachón and Tinaja caves. In Pachón we recovered an OD_H = 2.28 mm (s.d. 0.24 mm), and OD_L = 2.71 (s.d. 0.24 mm). For Tinaja de OD_H = 2.35 mm (s.d. 0.35 mm), and

Table 1. MANCOVA made with GM body shape data. The asterisk is for statistically significant p-values.

	DF	Pillai	F	p
Population	3	1.97	17.12	> 0.001*
Centroid Size	1	0.13	1.28	0.249
Pop*CZ	3	0.42	1.48	0.139
Residuals	37			

Table 2. Average of the Orbit diameter height (OD_H) and length (OD_L), and their respective proportions with respect to the fish's Standard Length (SL) and Head Length (HL). All the measurements are presented in mm. The standard deviation is presented for each measurement.

	Refugio	S.D.	Pachón	S.D.	Tinaja	S.D.	Florido river	S.D.
SL	45.42	9.61	51.83	1.93	58.79	7.98	58.30	8.20
OD_H	2.35	0.77	2.28	0.24	2.35	0.35	5.11	0.81
OD_L	2.87	0.73	2.71	0.24	2.88	0.39	5.13	0.86
HeadL	11.90	2.32	12.62	0.68	14.02	1.57	13.72	2.29
OD_H/SL	0.05	0.01	0.04	0.00	0.04	0.00	0.09	0.01
OD_L/SL	0.06	0.01	0.05	0.01	0.05	0.01	0.09	0.01
HeadL/SL	0.26	0.02	0.24	0.02	0.24	0.01	0.23	0.01
OD_H/HL	0.20	0.06	0.18	0.02	0.17	0.02	0.37	0.03
OD_L/HL	0.24	0.05	0.22	0.02	0.21	0.02	0.38	0.03

OD_L = 2.88 mm (0.39 mm). As expected, the orbit diameter values were higher in the surface population (Table 2).

The individual dot at the surface boxplot corresponded to the biggest fish (SL = 68.2 mm, with an OD_H = 5.53 mm, and OD_L = 5.5 mm), while for the El Refugio the individual dot corresponds to a surface-like fish, with completely functional eyes (SL = 70.6 mm, with an OD_H = 4.24 mm, and OD_L = 4.49 mm, Suppl. material 2).

Discussion

Since the discovery of the first cave population of *Astyanax mexicanus* in 1936 at La Cueva Chica, the history of cave explorations in the Sierra de El Abra has been extensive, with an important increase in the number of caves discovered during the 70's, as described in Mitchell et al. (1977) and Elliott (2018). In recent years (Espinasa et al. 2018; Espinasa et al. 2020), new cave populations have been discovered within southern Sierra de El Abra system, which has increased our understanding of its limits and connectivity between cave and surface populations.

The present study is an example of this, where the discovery of the El Refugio cave, has increased the number of populations in the northern part of Sierra de El Abra *sensu* Elliott (2018). Before this study, only Pachon and Venadito caves were known to harbor troglomorphic fish. The distance between El Refugio cave and Arroyo Santa Rita is 1.7 km, and the vertical height is 40 m, which makes it possible for surface fish from Arroyo Lagarto to swim upstream (rheotaxis) and reach the cave's entrance. The distance between El Refugio cave and Pachón cave is 4.5 km.

Some of the caves in the Sierra de El Abra, Sierra de Guatemala, and Micos mountain ranges capture surface streams, so these caves play the role of active sinkholes in the karst landscape of these regions. Nevertheless, Pachón and El Refugio caves behave differently, that is, they are resurgences where water flows out of the cave, joining the surface streams. For the Pachón cave, it has been hypothesized that the main connection with the surface drainage is given only in heavy rainy seasons when the cave's

water overflows present as a resurgence and the subterranean water achieves surface streams creating a sporadic path for surface fish to get into the caves (Bradic et al. 2012; Espinasa and Espinasa 2016).

Previous studies have suggested a temporal connection between the Pachón cave and Arroyo Lagartos (Bradic et al. 2012), from the former to the latter. Thus, during wet years, the Pachón entrance is a resurgence, creating a path for connecting surface fishes from Arroyo Lagartos. The nearest branch of Arroyo Lagarto is only 1 km away, but there is a 50 m vertical distance between the stream and the cave's entrance. In this regard, in 1991 some individuals with variable eye sizes and melanin pigmentation were observed in Pachón cave (Langecker et al. 1991), suggesting surface fish gained access to the cave. In El Refugio cave, we observed a mixed population, with a great variation in the pigment and eye loss, from the completely pigmented and eyed fish to the albino with complete eye loss, suggesting that this population maintains a much higher gene flow with the Arroyo Lagartos surface population than Pachón cave does.

In this regard, the Sierra de El Abra formation corresponds with a Cretaceous marine rock deposited in the platform, basin paleoenvironments (Agua Nueva, San Felipe, and Mendez formations), and Quaternary igneous rocks and alluvial deposits (Elliott 2018). The oldest unit cropping out in the area is the Sierra de El Abra formation, which is a succession of reef limestones and dolomites deposited during the Albian-Cenomanian system (Aguayo-Camargo 1975). The Agua Nueva Formation is composed of interbedded carbonaceous shale and limestone rocks of the Turonian age (Carrillo-Bravo 1971). The San Felipe formation comprises an alternation of limestones, marlstones, and tuffs. The age of this unit has been determined through paleontological studies as Coniacian-Santonian (Muir 1936; López-Ramos 1979). The Mendez formation has been described as a Late Cretaceous (Campanian-Maastrichtian) succession of shale, marlstone, and scarce sandstone and limestone (Carrillo-Bravo 1971; Keller et al. 1997).

The history of this area began in the Late Cretaceous and extends to the Eocene (Fitz et al. 2018), this deformational event creates a typical anticline-synclinal structural arrangement that dominates de geomorphology of the region. Several caves in the area, including the El Refugio and Pachón caves, were developed near the contact between the Agua Nueva and San Felipe formations. This geological contact probably acts as an inception horizon (Filipponi et al. 2009) (Fig. 3A, B). The karstification develops through the Agua Nueva formation and then it is limited due to the lower permeability of the San Felipe formation rocks.

The probability that a surface fish can get into these two caves (i.e. Pachón and El Refugio) is different since for the Pachón cave the entrance is at an altitude of 203 masl and corresponds to a perched system on a steep hill (Suppl. material 3: fig. A), being the base level of the valley at 175 masl, thus, corresponding with a geographic barrier that can only be avoided during important floodings. On the contrary, the El Refugio cave entrance is at 229 masl, and at the same base level as the temporal surface stream (Río Lagartos), thus, it is likely that every rainy season surface fish have direct and easy access to El Refugio cave.

By other hand, the subterranean connectivity between these two caves nowadays seems unlikely, since, between Pachón cave and El Refugio cave, there is a fossil canyon

of 100 m deep (Suppl. material 3: fig. B), which can act as a barrier. Espinasa and Espinasa (2016) argued that as tectonism uplifted the El Abra region, the limestone eroded, changing the course of the rivers. Around one to four million years ago, the Río Comandante, which ran through the aforementioned canyon, changed its course north, to its present position in La Servilleta canyon. There are several of these fossil canyons in the Sierra de El Abra and they have been shown to be considerable biogeographical barriers to the dispersal of aquatic organisms (Espinasa et al. 2020). Further studies evaluating the gene flow between these two cave systems can give additional information about their present and historical connections.

The geometric morphometric analyses showed very interesting results, with some individuals from El Refugio cave being clustered with Tinaja cave, while two individuals analyzed from the El Refugio fish were assigned to the Pachón cave cluster. Based on the mitochondrial data, we recovered haplotype A for the El Refugio cave, which is the same haplotype found in Pachón cave, and different from Tinaja cave. Previous studies have shown the Pachón cavefish exhibit a disparity between nuclear and mitochondrial data, with the nuclear data clustered with the rest of the populations from the Sierra de El Abra, while the mitochondrial haplotypes clustered with the nearby surface fish (Dowling et al. 2002; Strecker et al. 2004, 2012; Herman et al. 2018). The presence of the same haplotype between surface and Pachón cavefish has been suggested as the result of a recent episode of introgression with surface fish (Langecker et al. 1991). In 1986, individuals with variable eye sizes and melanin pigmentation were observed in the former albino and eye-reduced Pachón population (Langecker et al. 1991). We suggest a mitochondrial introgression from the surface population to El Refugio cave, rather than shared ancestry (i.e., haplotype A), similarly to the patterns observed at Pachón cave (Dowling et al. 2002; Strecker et al. 2004, 2012; Herman et al. 2018), however, further studies considering the nuclear information are need to better understand the historical and recent connections among northern caves of the Sierra de El Abra (i.e., Pachón and El Refugio), and their relation with Los Sabinos area. Finally, it is important to highlight that this new cave population is highly vulnerable since there are important impacts and threats to this new population that are mainly human-derived. Principally, the well in the El Refugio cave system has a pipe from which water is extracted for local consumption, which potentially can lead to habitat destruction since it is not clear the extraction volume by this pipe. Another concern is that the cave is located within an urban settlement, which can affect the water quality of the wastewater discharged into the local aquifer. Finally, El Refugio cave gives access to only small pools with very small populations of at most tens of individuals. Thus, it is important to prevent overfishing by scientists or common people.

Conclusions

The Sierra de El Abra corresponds to a unique system in which we can find more than 200 caves, holding a great diversity of fauna (Mitchell et al. 1977). The *Astyanax mexicanus* cavefish have adapted to these cave environments, occupying 33 caves in the El Abra

region (Elliott 2016; Espinasa et al. 2021). El Refugio cave is a newly described resurgence cave inhabited by troglomorphic fish in the Northern Sierra de El Abra. The new population may be isolated from its nearest cave, Pachón cave, due to a fossil canyon. This new population corresponds to a mixed population of cavefish, surface fish, and intermediate individuals in morphology, due to intermittent contact with the Arroyo Lagartos surface population, as evidenced by the phenotypic and mitochondrial data. This discovery opens the possibility of describing the historic and recent events of cave colonization from their surface ancestors. But most important, it will allow for a better understanding of the evolutionary history of Pachón cave, and the Northern part of the Sierra de Abra.

Acknowledgements

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Supplementary material 1

DNA sequence of the mitochondrial 16S rRNA

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Data type: Nucleotide differences between lineages

Explanation note: El Refugio cavefish had identical sequences to fish from Pachón cave and from the local surface *Astyanax mexicanus* (Haplotype A). Compared to the mitochondrial haplotype B of cavefish from Sabinos, Tinaja, and Curva, they differed by 5 bp.

Link: https://doi.org/10.3897/subtbiol.45.98434.suppl1

Supplementary material 2

Geometric morphometric coordinates, PCA values obtained from the geometric morphometric data and orbit diameter values for the populations

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Data type: Morphological data

Explanation note: SM2_T1. Geometric morphometric coordinates. SM2_T2. PCA values obtained from the geometric morphometric data. SM2_T3. Orbit diameter values for the populations.

Link: https://doi.org/10.3897/subtbiol.45.98434.suppl2

Supplementary material 3

Contrasting vertical isolation between Pachón and El Refugio caves and the fossil canyon between Pachón and El Refugio caves

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Data type: Geographic data

Explanation note: **A**) Contrasting vertical isolation between Pachón and El Refugio caves. In blue are the temporal streams, source of surface fish. Arrows point downstream. The arrow's base is at the spring where the stream is born during the rainy season. Pachón is perched on a steep hill, so it is difficult to access by surface fish. Refugio cave on the contrary is at the same base level as the surface temporal stream and thus has easy access for surface fish to hybridize with the troglomorphic population. **B**) The fossil canyon between Pachón and El Refugio caves. The Río Comandante, used to ran through this fossil canyon but changed its course to the north, to its present position at La Servilleta canyon.

Link: https://doi.org/10.3897/subtbiol.45.98434.suppl3

RESEARCH ARTICLE

A new genus, *Tuberocandona* gen. nov. (Crustacea, Ostracoda, Candonidae) and past to present ostracod species diversity in Texas (USA)

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Abstract

A new ostracod genus, *Tuberocandona* **gen. nov.**, was collected from Honeycut Hollow Springs, Texas, USA Morphological comparisons and cladistic analyses showed that the new genus displays several different features (e.g. presence of two tubercules on each of the valves, numbers of A1 segments, shape of A2 claws, shape and presence of two claw-like setae on the clasping organs, absence of d2 and dp setae on T2 and T3, absence of alpha and beta setae on Md, shape of hemipenis) from other genera of the tribe. Including the new species, the number of non-marine ostracods known from inland waters of Texas is now 118 species in 45 genera. With the aim of documenting ostracod biodiversity in Texas (USA) by including fossils, we sought documents published from 1927 to 2022 and were able to list 673 ostracod taxa belonging to 142 genera. Among the fossils, 73 ostracods were the oldest records during the Pennsylvanian period (ca. 310 mya), while there were only 42 taxa reported from the Holocene. The Eocene had the highest number of ostracods (126 taxa). In comparison, the living species had only 18 of 673 taxa that were considered nonmarine forms. There are only six species in common with the fossils and recent records. These results suggest the potential for relatively high ostracod species richness and diversity in Texas. This is indeed strongly supported by the present study and the described new genus and its type species (*Tuberocandona leonidasi* **sp. nov.**).

Keywords

Cladistic analyses, diversity and distribution, Nonmarine Ostracoda, Rheocrene spring

Introduction

Over the last ten years or so, studies on the inland water ostracods of Texas have provided interesting results that highlight unique and high species diversity in the state (Külköylüoğlu et al. 2021; Külköylüoğlu and Tuncer 2022). These studies, in total, revealed about 117 ostracod species. Comparing the Texas ostracod list to that of the USA shows Texas contains about 25% to 33% of the USA total (Külköylüoğlu, pers. obs.). Moreover, this number is also relatively high compared to many other countries in the world such as Turkey (>160 spp.) (Külköylüoğlu, unpublished data), Italy (156 spp.) (Pieri et al. 2015, 2020), China (154 spp.) (Yu et al. 2009), India (152 spp.) (Karuthapandi et al. 2014), and Germany (126 spp.) (Frenzel and Viehberg 2005). Hence, considering the areas and habitats not sampled and/or not studied yet, the State of Texas will probably contain a much higher species diversity than what we have already discovered. Indeed, contemporary studies in different taxonomic groups (Gibert et al. 1990; Bowles and Arsuffi 1993; Hall et al. 2004; Segers 2008; Hutchins et al. 2020; Gibson et al. 2021) also support this view that the area has high species diversity including epigean, subterranean and groundwater habitats. Studies on the fossil ostracods revealed similar results and even much higher species diversity. These preliminary findings have made us ask how fossil (past) and present (contemporary) non-marine ostracod assemblages and diversity are related. This question is important for at least three reasons: i) aids in understanding the level of correlations between the fossil and live ostracod assemblages, ii) helps to explain how and why replacement of some taxonomic group(s) occurred (if it did) in time, and iii) provides a framework when describing a new species and genus amid other taxa. Inquiring into the literature available, there are no comparative analyses between fossil and current ostracod diversity in Texas. The aims of the present study are 1) to propose a new species and genus *Tuberocandona leonidasi* gen. nov. sp. nov., and 2) to compare and correlate fossil and current numbers of ostracods in the State of Texas (USA).

Methods

Site description

This new species of ostracod was collected from a spring on the privately owned C.L. Browning Ranch in eastern Blanco Co., Texas (Fig. 1). Honeycut Hollow Spring forms the headwaters of Honeycut Creek, which terminates at the Pedernales River during flood events. The flow from this spring returns to the ground around 500 meters downstream (Brune 1981). Abiotic parameters were measured using a Hydrotech compact DS5 with averages calculated for water temperature (22.2 °C), dissolved oxygen (3.1 mg/l), pH (6.6), and electrical conductivity (651 µS/cm) collected during six visits from 13 July to 23 August 2021. The main spring is small, a little over a meter in circumference. This orifice flows out from under what appears to be a bedding plane with maidenhair fern (*Adiantum capillus-veneris* Linneaus, 1753) along the top edge.
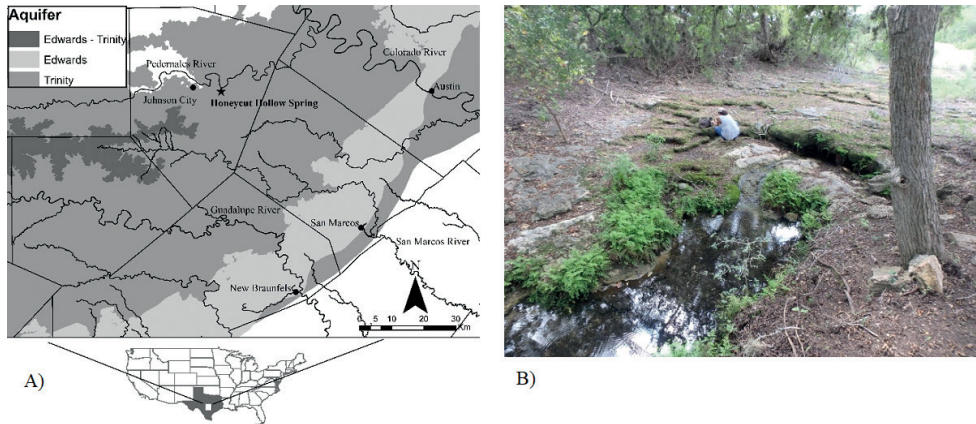
Figure 1. A location (*) of sampling site in Texas, USA. **B** detailed photograph of Honeycut Hallow Spring.

Other smaller seeps emerge from along the bottom of this bedding plane. The spring outflow is mainly smooth bedrock; however, gravel substrates are available within the spring opening.

Honeycut Hollow Spring was sampled by placing a 150 μm mesh drift net over the main orifice. The net was lodged into the orifice and surrounded by cobble to maintain the net in place and checked weekly. Samples collected were stored in 95% ethanol and returned to the laboratory where sorting the ostracods from the material was done under magnification removing all aquifer taxa. Sorted samples (e.g. *Stygobromus* sp. (Amphipoda), *Lirceolus* sp. (Isopoda), *Phreatodrobia* cf. *nugax* (Pilsbry and Ferriss 1906) (snail), and harpacticoid copepods)) were also stored in 95% ethanol.

Methodology

Using fine needles, individual species were separated from each other under the SZ-X7 Olympus stereomicroscope. We deposited ostracods in 70% ethanol. Species identification was determined after dissecting adult specimens (i.e., taking the individual specimen to the slide with a glass pipet, measuring the individual, separating soft body parts from the carapace and dissecting the soft parts in lactophenol solution) under a light microscope (Olympus BX-51). Each sample was preserved with a cover slide and labeled with the catalogue number, name, and was stored in the laboratory collection. Line drawings of the soft body parts were made with a camera lucida attached to the light microscope. Scanning Electron Microscope (SEM) was used to take photographs of the carapace and valves at the Department of Geology, Hacettepe University. These samples were kept on the SEM stubs. Although not limited, we generally used common taxonomic keys (e.g. Meisch 2000; Karanovic 2012) for the species identification. During which, the chaetotaxic scheme for the A2 proposed by Martens (1987) and the terminology of the legs were used after Broodbakker and Danielopol (1982) and Meisch (2000). We keep the samples at the Limnology Laboratory of the Biology Department, Bolu Abant İzzet Baysal University, Bolu, Turkey.

Clustering analyses

To determine similarities among the genera of the subfamily Candoninae, we used statistical package program NONA and WinClada, version 1.00.08 (Nixon 2002). During the analyses, including the new genus (*Tuberocandona* gen. nov), a total of 50 genera of the subfamily and two outgroup genera (*Cypria*, *Cyclocypris*) (total 52 genera) were ran to compare 36 morphological characteristics due to their taxonomic importance (Appendix 1) (see Karanovic 2007, 2012; Külköylüoğlu et al. 2021). After coding the characters in the data matrix, they were weighted for calculating indice values of consistency (Ci) and retention (Ri). Heuristic and Rachet Island Hopper were used to provide the best fit. This includes 300 replications; 1 best tree to hold, 3 characters to sample, 10 random constraint levels and amb-poly, along with a tree bisection and reconnection method of branching-swapping (Nixon 2002; Karanovic 2007).

Abbreviations: A1, first antenna; **A2**, second antenna; **G1–G3** and **GM**, **Gm**, claws on A2; **H**, height; **L**, length; **LV**, left valve; **Md**, mandibula; **Mxl**, maxillula; **RV**, right valve; **T1**, first thoracopod; **T2**, second thoracopod; **T3**, third thoracopod; **UR**, uropod; **W**, width.

Results

Based on the published information (e.g. articles, reports, theses, notes) in Texas, 673 fossil ostracods belonging to 142 genera were reported in the literature between 1927 and 2022 (Table 1). While 655 taxa belong to the marine taxonomic groups, there are 18 ostracod species (Table 2) considered as nonmarine. Six of the 18 non-marine species exist both in fossil and living populations. The oldest 74 fossil ostracod species aged about 310 mya were reported from the Pennsylvanian period (Paleozoic era) while there were about 42 taxa from the Holocene (Table 1). Between them, there were more than 500 taxa distributed among the periods, except Neogene and Triassic (possibly due to lack of data). The highest number of fossil ostracods with 126 taxa were encountered from the Eocene. In contrast to the total of 142 fossil genera, by 2022 only 44 non-marine genera were described from Texas. In addition to these 44 genera, during the present study, we herein propose to recognize an additional genus of non-marine ostracod from Texas, namely (*Tuberocandona* gen. nov.). It is represented by one species (the type species (*Tuberocandona leonidasi* sp. nov.)) (Figs 2–4) with a living population at Honeycut Hollow Spring. The new genus belongs to the tribe Cabralcandonini and portrays clear morphological differences from its other congeners (Fig. 5). Accordingly, this elevates the total number of non-marine ostracods known from Texas to 118 species in 45 genera.

Results indicate that (i) ostracod species diversity is actually and potentially very high in Texas, (ii) most of the fossil taxa belonged to marine ostracods that supports

Table 1. Numbers of fossil taxa reported from 1927 to 2022. Note that the sum of 764 taxa is listed here because several ostracods were reported more than once in different periods or epochs. There are total of 673 single taxa reported once from the state.

Period/epoch	Numbers of occurrence
Holocene Epoch	42
Pleistocene Epoch	6
Early Pleistocene	3
Late Pleistocene	1
Pliocene Epoch	3
Miocene Epoch	8
Oligocene Epoch	4
Eocene Epoch	126
Middle Eocene	70
Paleocene Epoch	15
Cretaceous-Tertiary Period	83
Cretaceous Period	61
Upper Cretaceous	105
Lower Cretaceous	97
Upper Jurassic	15
Middle Permian	51
Pennsylvanian Period	74
Total	**764**

Table 2. A total of 18 nonmarine fossil ostracods reported from different epochs/periods in Texas. Sources: 1, Swain (1999); 2, Maddocks (1988); 3, Peck (1941); 4, Artusy (1960); 5, Swain (1955); 6, Roth (1933); 7, living forms are known; 8, synonym of *Fabaeformiscandona obtusa* (Bronstein, 1947).

Taxa	Epoch/period	Source
Candona sp.	Early Pleistocene	1
Cyprideis sp.	Early Pleistocene	1
Limnocythere sp.	Early Pleistocene	1
Candona rangliensis	Eocene	1
Cyprideis salebrosa[7]	Holocene	2
Chlamydotheca llanoensis[7]	Late Pleistocene	1
Candona sp. indet.	Lower Cretaceous	3
Limnocythere sp.A	Middle Eocene	4
Hemicythere conradi	Miocene	5
Cypricercus? sp.1	Oligocene	1
Darwinula sp.	Oligocene	1
Candona rawsoni[7,8]	Pleistocene	1
Cyprideis torosa[7]	Pleistocene	5
Limnocythere sanctipatricii[7]	Pleistocene	5
Cypridopsis vidua[7]	Pleistocene	5
Cyprideis locketti	Pliocene	5
Darwinula aurera	Pliocene	5
Pseudocypridina piedmonti	Upper Jurassic	6

high richness and species diversity, and (iii) the ratio of living/fossil ostracods (118/673) pinpoints the need of specific attention on the living non-marine ostracod fauna. This is an especially important issue for conservation programs future planning.

Taxonomy

Class: Ostracoda Latreille, 1802
Subclass: Podocopa Sars, 1866
Order: Podocopida Sars, 1866
Suborder: Cypridocopina Baird, 1845
Superfamily: Cypridoidea Baird, 1845
Family: Candonidae Kaufmann, 1900
Subfamily: Candoninae Kaufmann, 1900
Tribe: Cabralcandonini Külköylüoğlu et al., 2019

Genus: *Tuberocandona* gen. nov.
https://zoobank.org/7483A624-5331-41C9-9253-65CEA8E35E5A
Figs 2–4

Genus diagnosis. Carapace sub-rectangular with two tubercules or nodes on each side (diagnostic character) and surface ornamented with deep hexagonal and pentagonal cells covered with dense spines. Both marginal zones with dense spines. Tubular pore canals with a short sensory seta (diagnostic character), aperture lobate. LV overlaps RV on all sides. Hinge adont. Five adductor muscle scars, one frontal and one mandibular scars visible in about the center of the valves. Inner lamella wide at both ends. LV with an anteroventral node. Selvage absent. A1 6-segmented. Rome and Wouter's organs absent. A2 4-segmented, y1–2 and swimming setae absent. t-setae not transformed in the male. z1-seta absent in males, z2 seta present. Mandibular palp 4-segmented; alpha and beta setae absent (see discussion). Second segment with 4 setae internally, and two setae externally. Third segment with a thin slightly plumose gamma seta. Terminal segment slightly rectangular with one fused claw and one claw-like seta. Maxillula with three endites and two (I–II) segmented palp. Claws on third endite not bristled. Terminal segment of Mxl-palp subsquared. First thoracopod symmetrical in female but transformed into prehensile palps in male. Walking leg (T2) 5-segmented with "d1" seta on basal segment. Cleaning leg (T3) 5-segmented with "d1" seta present. Terminal segment with one long, one medium and one short seta. Uropod well developed with anterior and posterior claws and anterior seta, posterior seta absent. Genital lobe in female rounded without appendages. Zenker organ with 5 whorls. Hemipenis large with outer lobe (lobe a) oval, inner lobe (lobe b) rounded, and large medial lobe (lobe h) subtriangular.

Type species. *Tuberocandona leonidasi* sp. nov. Külköylüoğlu, Ataman, Gibson, Diaz.

Derivation of name. A word with Latin origin "tubero", meaning "tubercle, lump, node", is combined with the genus name Candona (gender feminine) due to presence of two tuberculated (noded) alae type of structures on both sides of the carapace.
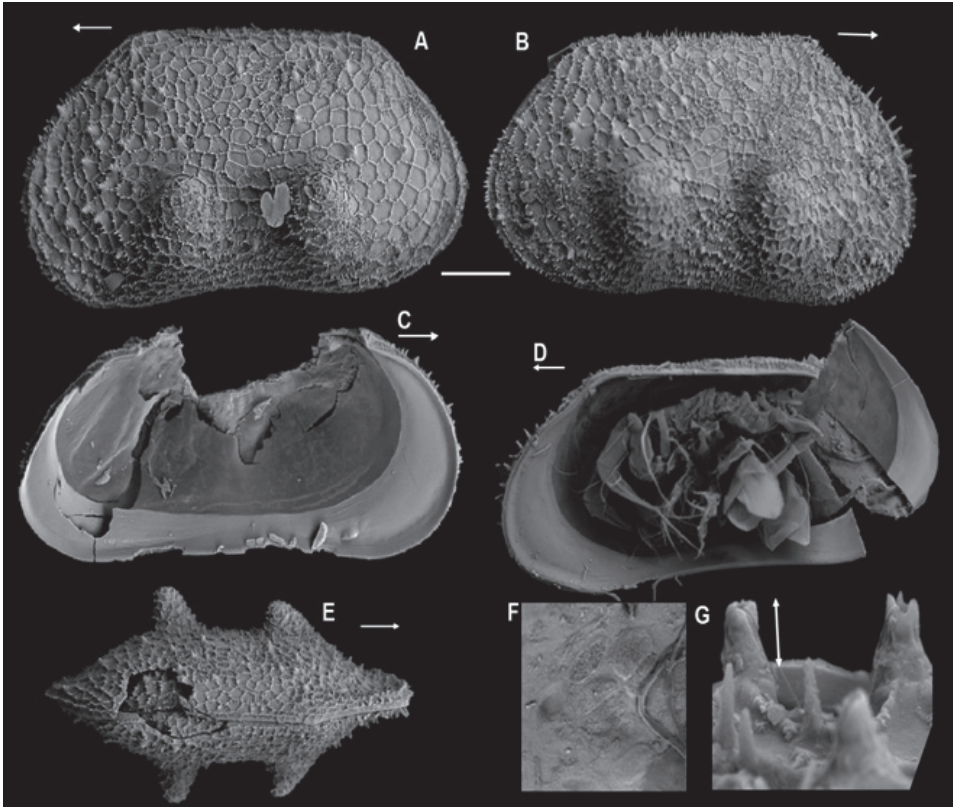
Figure 2. *Tuberocandona leonidasi* sp. nov. **A** LV external view ♂ (Holotype) **B** RV external view of ♀ (Allotype) **C** LV internal view of ♀ (Allotype) (dorsal margin broken) and **D** RV internal view of ♂ with hinge (Paratype) (posteroventral margin broken) **E** dorsal view of ♂ (Paratype) **F** muscle scars of ♂ **G** tubular pore canal of ♂. Scale bars: 70 μm (**A–E**); 10 μm (**F, G**) (two-sided arrow).

Tuberocandona leonidasi sp. nov.

https://zoobank.org/7A749087-625F-4A7C-95A6-C6490CBDBDD3

Diagnosis. *Holotype.* Adult ♂ dissected in lactophenol solution with soft body parts (no: OK-TX-BCo-1) sealed with translucent nail polish; valve kept on a micropaleontological slide (no: OK-TX-BCo-2). Collected from the type locality on 4 and 9 of August 2021 by Peter Diaz.

Allotype. Adult ♀ dissected in lactophenol solution with soft body parts from the type locality (no: OK-TX-BCo-3). Collected by Peter Diaz.

Paratypes. Two ♂ (OK-TX-BCo-4) and two ♀ (OK-TX-BCo-5) mounted and sealed in glass slides, collected from the type locality; total of seven ♀ and four ♂ collected from type locality.
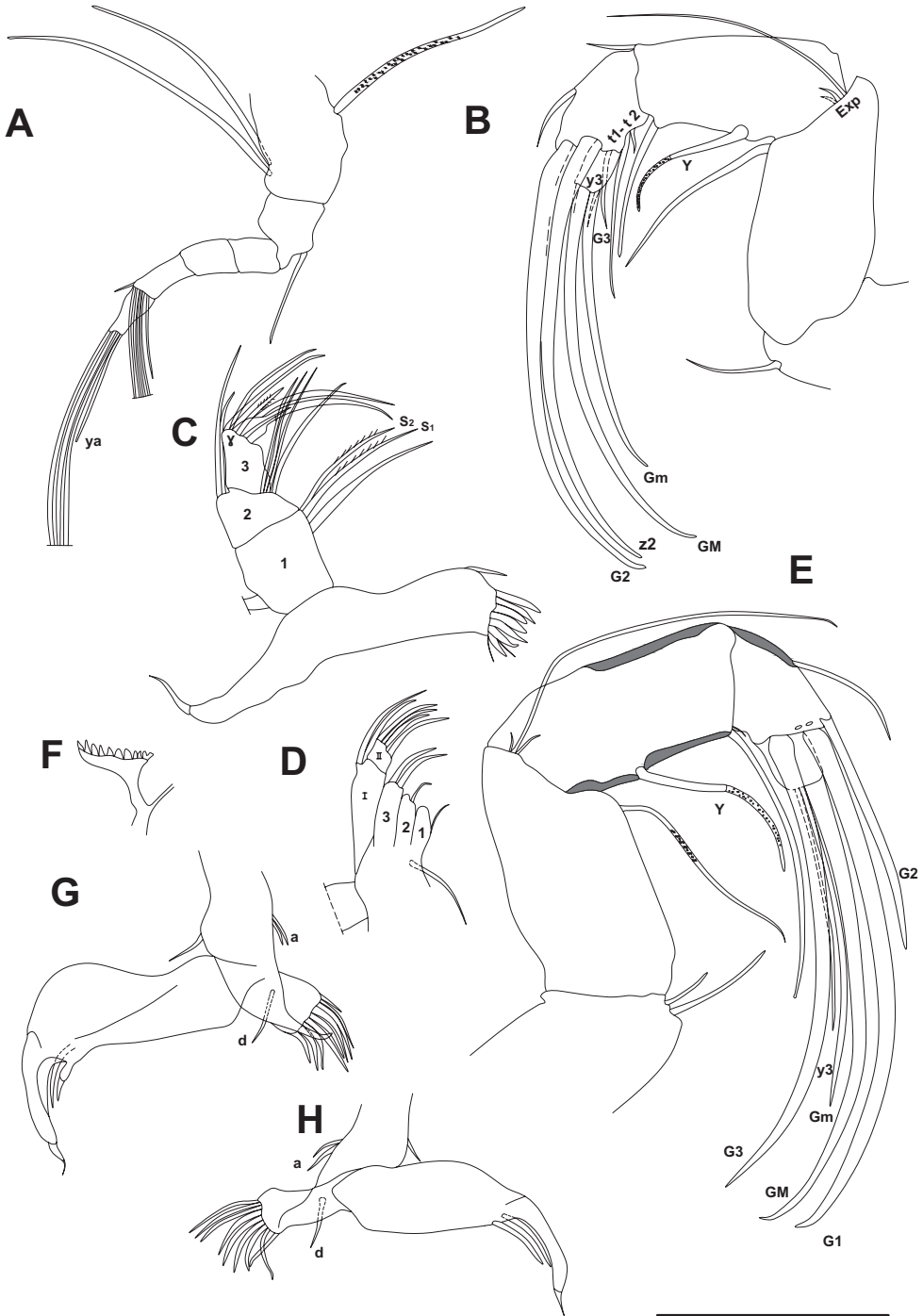
Figure 3. *Tuberocandona leonidasi* sp. nov. **A** A1 **B** A2 **C** Md **D** M×1 **E** A2 **F** rake-like organ **G** right clasping organ **H** left clasping organ. **A–D, F–H** ♂ (Holotype); **E** ♀ (Allotype). Scale bar: 10 μm.
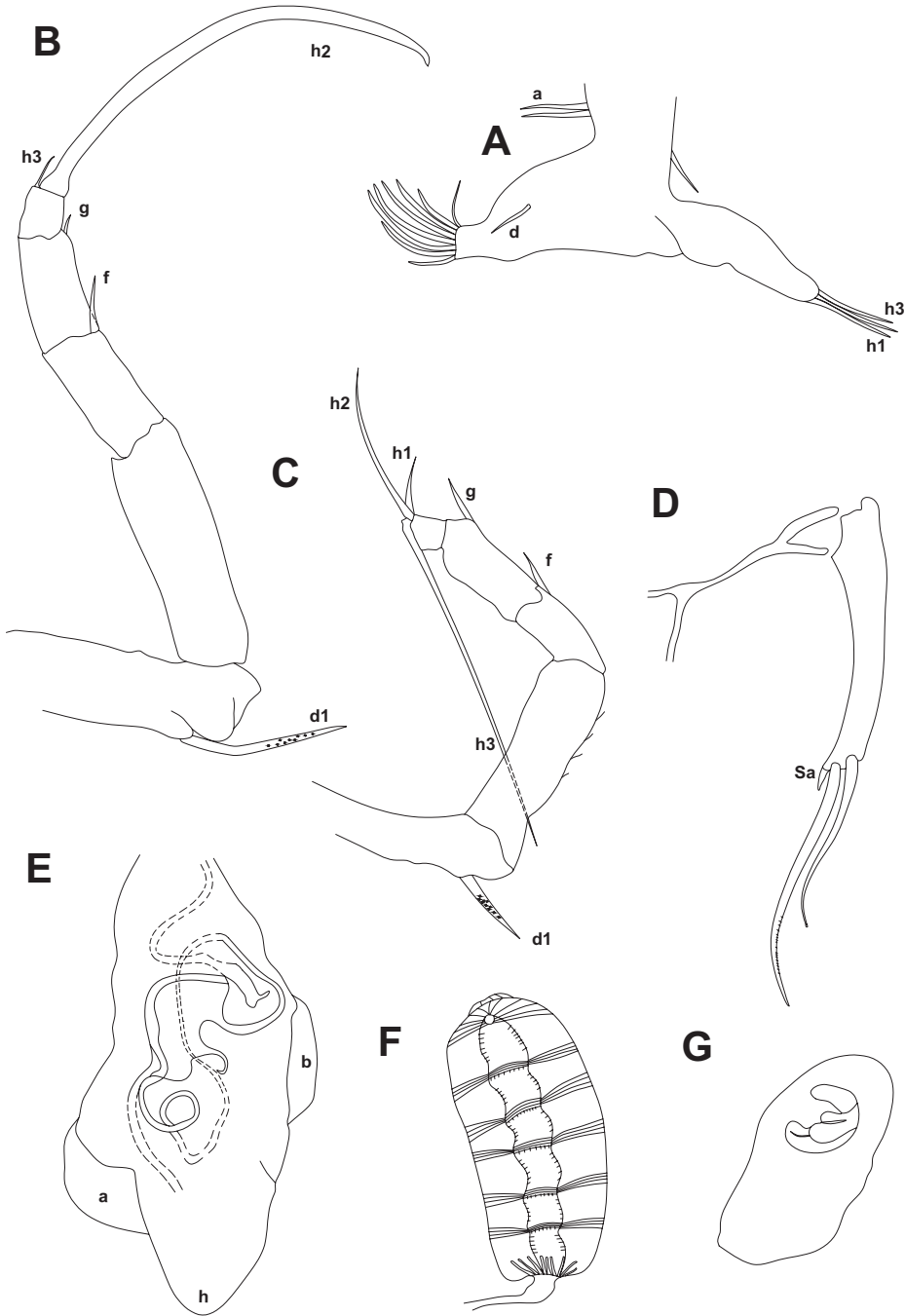
Figure 4. *Tuberocandona leonidasi* sp. nov. **A** T1 **B** T2 **C** T3 **D** uropod and uropodal attachment **E** hemipenis **F** Zenker's Organ **G** genital organ. **A, G** ♀ (Allotype); **B–F** ♂ (Holotype). Scale bar: 10 μm.

Type locality. Perennial headwater of Honeycut Hollow Spring, Blanco County, Texas, USA (30.266319, -98.333497).

Derivation of name. The species is named after the original landowner Caleb Leonidas Browning, Jr as per the current landowner's suggestion.

Description. Male: *Measurements* (based on midlength). L=0.51–0.56 mm, H=0.24–0.27 mm, W=0.20–0.28 mm (*n*=4). LV overlapping RV anteriorly and posteriorly (Fig. 2A, D–G). Carapace elongate with two well-developed alae type tubercules or nodes on each valve, laterally. In dorsal view (Fig. 2E), both margins pointed. Carapace surface ornamented and covered with spines, especially around each hexagonal cells, pore canals tubular with a thin seta (Fig. 2G). Both margins with stiff spines (Fig. 2D). Dorsal margin almost straight. Calcified inner lamella smooth, without inner list, wide in both margins. LV with internal node anteroventrally, RV smooth. Four large and one small central and two ventral muscle scars located about the center between the tubercles (Fig. 2F). Eyes not visible.

Antenulle (A1): Six segmented (Fig. 3A): First segment (base) well-developed with a slightly plumosed long seta on dorsal margin, and two unequally long smooth setae on ventral margin. Second segment with a smooth dorsal-apical seta medium in size. Third and fourth segments without setae. Fifth segment with four long setae and one medium-sized antero-dorsal seta, and one very short ventral-apical seta. Terminal segment with three long and a medium-sized smooth aesthetasc ya (ca. 1/3 of long setae).

Antenna (A2): Four segmented (Fig. 3B). First segment with a long smooth dorsal-apical seta, reaching halfway of terminal segment. Exopod with one medium-sized and two very small exopodial setae. Second segment without natatory setae. Aesthetasc Y long with two segmented parts extending to end of subterminal segment, proximal part slightly longer than plumosed distal part. Anterior-dorsal seta smooth and long, anterior-dorsal seta very short (ca. ¼ of subterminal segment). Penultimate segment with one short seta in dorsal margin, t-1 seta very short, t2 seta long 2× terminal segment all smooth. t3–4 setae absent. Y2-seta not observed. G1 claw absent, G2 claw well developed, G3 claw very short and thin (ca. 2× of terminal segment). Seta z-2 claw-like long reaching tips of G2 claw. Setae z1 and z3–4 not observed (cf. female A2). GM and Gm claws well-developed on terminal segment, Gm claw short about 3/4 of GM, and y3-seta very short and thin about size of terminal segment. All claws and z1-setae smooth.

Mandible (Md) (Fig. 3C): Coxa with seven robust teeth and thin short setae internally, and dorsal seta short and stout. Palp four segmented; first segment with vibratory plate bearing six plumosed setae, S1 and S2 setae plumosed and unequally long, alpha seta not observed. Second segment with a bunch of four long smooth setae, beta seta not observed. Two (one long and one medium sized) unequally long external setae extending to tips of terminal segment. Penultimate (third) segment with two equally long and smooth external setae, two internal setae unequally long and smooth, gamma seta medium-sized and slightly plumosed. Terminal segment slightly rectangular fused with one claw and one seta-like claw. L:W ratio of terminal segment 1.2.

Maxillula (Mxl) (Fig. 3A): With three small endites and a two-segmented palp, vibratory plate with 12–13 plumosed setae. First, second and third endites with five, five and six setae similar in size (two bristles smooth), respectively. Base of first endite with one long and

slightly plumose seta. First segment of Mx-palp with 2 medial setae. First palp ca. 3× longer than terminal (second) palp. Second segment squarish with 4 claw-like and smooth setae.

Rake-like with 9–10 teeth (Fig. 3F).

First thoracic leg (T1) (Fig. 3G, H): Palps well developed and asymmetrical ending with hooked-like fingers modified into clasping organs. Right palp (Fig. 3G) stronger and robust. Left palp (Fig. 3H) slightly longer than right one. Both fingers ending with a well-developed spine, and two claws. Endite (masticatory process) with 9 to 10 smooth long setae (thicker than usually known). Two unequally long "a" and one "d" seta present, setae "b" and "c" not observed. Vibratory plate with one smooth short seta.

Second thoracic leg (T2) (Fig. 4B): Five segmented with a medium-sized slightly plumose d1 seta on the first segment. Second without seta. Third and fourth segments with unequally long f and g setae, respectively. Terminal segment subrectangular, seta h1 reduced or absent, h2 claw smooth and well-developed, longer than the last three segments. Seta h3 thin.

Third thoracic leg (T3) (Fig. 4C): Five segmented with a well-developed slightly plumose d1, setae d2 and dp absent. Second segment without seta e. Third and fourth segments with smooth "f" and "g" setae, respectively. Terminal segment square three h setae as seen in the Figure.

Uropod (Fig. 4D): Well developed ramus with anterior and posterior claws. Anterior seta short and spine-like, posterior seta absent. Both claws curved and slightly serrated anteriorly. Caudal attachment with one branch.

Hemipenis (Fig. 4E): Large and robust, outer lobe "a" rounded, inner lobe "b" small, medial lobe "h" large and slightly pointing.

Zenker organ (Fig. 4F): With five whorls ending with 15–16 sperm canal.

Color: Translucent to opaque white.

Description of female. Carapace similar in shape of male (Fig. 2B, C). Measurements: L=0.55 mm, H=0.25 mm, W=0.25 mm (*n*=2). G-claws (length ratio G1≈G3≈GM>Gm>G2) (G2 ca. 1/3 of G1) present on A2 (Fig. 3E). Setae z1–2 thin and very short slightly extending terminal segment, setae z3–4 not observed. Long seta on exopod reaching halfway of subterminal segment (cf. male exopod). Two unequally long and smooth setae present on basal segment (cf. male A2). T1 (Fig. 4A) normally developed, endopod with 2 short (h1, h3) setae, h2 seta not observed. All smooth. Endite with 8–10 apical setae, slightly transformed to claw like. Genital part (Fig. 4G) rounded with a robust genital hook inside. All other parts similar to the males.

Accompanying taxa. *Comalcandona tressleri* Külköylüoğlu and Gibson, and *Neglecandona* cf. *neglecta* (Sars, 1887).

Discussion

Ostracoda diversity in Texas

Contemporary studies on nonmarine ostracods (Külköylüoğlu et al. 2011, 2017a, b, c, d, e, 2019, 2021, 2022; Külköylüoğlu and Gibson 2018; Külköylüoğlu 2020;

Külköylüoğlu and Tuncer, in press), as well as other taxonomic groups (Reddell and Mitchell 1969; Hall et al. 2004; Segers 2008; Hutchins et al. 2020; Gibson et al. 2021), have clearly shown that the State of Texas contains highly unique species diversity and richness. However, knowledge about fossil fauna is not complete and there are gaps in the evolutionary record that need filling.

During the present study, we compiled all possible fossil ostracod taxa reported from 1927 to April 2022. The 673 fossil ostracod taxa from the Pennsylvanian to the Holocene periods strongly support the view that the area is of high diversity. Among the fossil taxa, there are only 18 ostracods classified as nonmarine (cf. nonmarine ostracod species list of Meisch et al. 2019). Moreover, only six of the 18 (Table 2) still have populations living in aquatic habitats today. Based on earlier studies (Delorme 1991; Forester 1991; Külköylüoğlu 2003), these six species are known to have relatively high ecological tolerances to different environmental variables. All are taxonomically well-known species with records from the Pleistocene to the Holocene (i.e. to the present). The other 12 taxa either do not currently exist or have not been identified at the species level.

During the present study, we found (see Tables 1, 2) that the ratio of the recent taxa in the total numbers of fossils (18 / 673 = 0.026) has increased since the last known nonmarine ostracod was reported from Holocene (Maddocks 1988), whereby the current ratio is now (118 / 673) increased to 0.175. Such an increase in nonmarine ostracods is apparently related to increasing numbers of studies since the 1930s. However, finding more nonmarine taxa from the Pleistocene to Holocene may also be explained by the fact that most of Texas laid beneath marine waters. Indeed, during most of the late Cretaceous (ca. 140 mya), much of Texas laid beneath marine waters when nonmarine ostracods were not able to establish in the area. Subsequently, nonmarine ostracods appear to have flourished after the intrusion of freshwater habitats (e.g. rivers, springs, underground waters). Finding 122 fossil taxa representing the Eocene Epoch corresponds to this period when freshwater habitats were appearing. According to Salinas et al. (2020) Honeycut Hollow Spring has a relatively stable discharge and long groundwater residence time with a deep flow path. The spring is located on the Cow Creek and Glen Rose limestone formations which is about 115–105 million years old (Young 1974; Barck 1992). The authors stated that water isotope (e.g. deuterium) values were close to constant, indicating that the spring did not respond to precipitation. In other words, the spring has not been affected by environmental changes (e.g. temperature fluctuations) and has been flowing continuously (Salinas et al. 2020). *Tuberocandona leonidasi* gen. nov. sp. nov. appears to be locally adapted to relatively stable aquatic conditions and is possibly endemic to this formation. This is especially important for paleontological studies that aim to explain the past historical environmental conditions. Karanovic (2007, 2012), working on variety of subterranean waters of Australia, postulated that such waters (i.e., springs, underground waters, and/ or spring related waters) can carry endemic populations even above the species level. Similar findings are also known for the members of Candoninae reported from South and Central America (Broodbakker 1983), Africa (Martens 1992) and North America (Texas) (Külköylüoğlu et al. 2017a, b, c, d, e).

Taxonomic comments

Cladistic analyses (Fig. 5) illustrated that *Tuberocandona* gen. nov. belongs to the tribe Cabralcandonini but with different features from the other genera of the subfamily Candoninae (Appendix 1). To minimize redundancy, discussion below focuses on those important diagnostic characteristics of the genus and the type species. Therefore, following features are subjected for discussion.

Carapace ornamentation, shape, and pore opening

Presence of two tubercules on each of the valves and spines on the carapace surface along with hexagonal and pentagonal ornamentation are totally unique to the genus. Although it is very common in marine ostracods, several species/genera of the subfamily portray different ornamentations on the carapace; for instance, there are fine longitudinal striations in the *Undulacandona* reported from groundwater located nearby Lake Biwa in Japan (Smith and Kamiya 2015). In addition to the pits, fine reticulations (cf. *Paracandona*) (Karanovic 2012), wrinkle-shaped ornamentations (see *Rugosuscandona*, *Cabralcandona*) (Külköylüoğlu et al. 2017b, 2019), and even bump-shaped ornamentations (see *Ufocandona*) along with variety of microreticulations (Külköylüoğlu et al. 2017e) are known. However, the formation of tubercules with dense spines such as occur in *Tuberocandona* gen. nov. are not known in the subfamily Candoninae where the carapace is mostly described as smooth, pearly lustre, and/or translucent in appearance (Meisch 2000; Karanovic 2004). According to Liebau (1977), macroreticulations may indicate early evolutionary stages of the taxonomic group. During which, macroreticulation can be reduced and the carapace may become smooth. If this is true, with dense spines and ornamentations *Tuberocandona* gen. nov. may represent one of the oldest lineages of the subfamily. Since it is a new description with limited knowledge about the species, generalization may not be possible now.

As stated above, the carapace shape of the new species has interesting outlines and is probably a good proxy for the adaptation to the groundwater environments. It is argued that if a species has rectangular and(or) triangular carapace shape with the posteroventral margin pointed, it most likely lives in relatively stable aquatic habitats where flow rate is low. This is the case for some Candoninae species (Pipík and Bodergat 2005, 2007; Külköylüoğlu et al. 2021). On the other hand, species with subcircular or oval shape are usually encountered in unstable conditions where fluctuations (e.g. flowing rates, evaporation, water movement actions) in the water body may occur. The shape of *Tuberocandona leonidasi* sp. nov. (rectangular shape with wide tubercles) suggests that its habitat (underground water body in the sampling site) has relatively stable conditions.

Pore openings are unique and differ from other congeners of the tribe Cabralcandonini and other members of the subfamily. Numbers of openings seem to be less than many other species. However, its normal pore openings may be longer (range 7–10 μm) than many other species. For example, in *Rugosuscandona scharfi*, height of the canal was between 0.25 and 0.30 μm (Külköylüoğlu et al. 2017b). Also, the
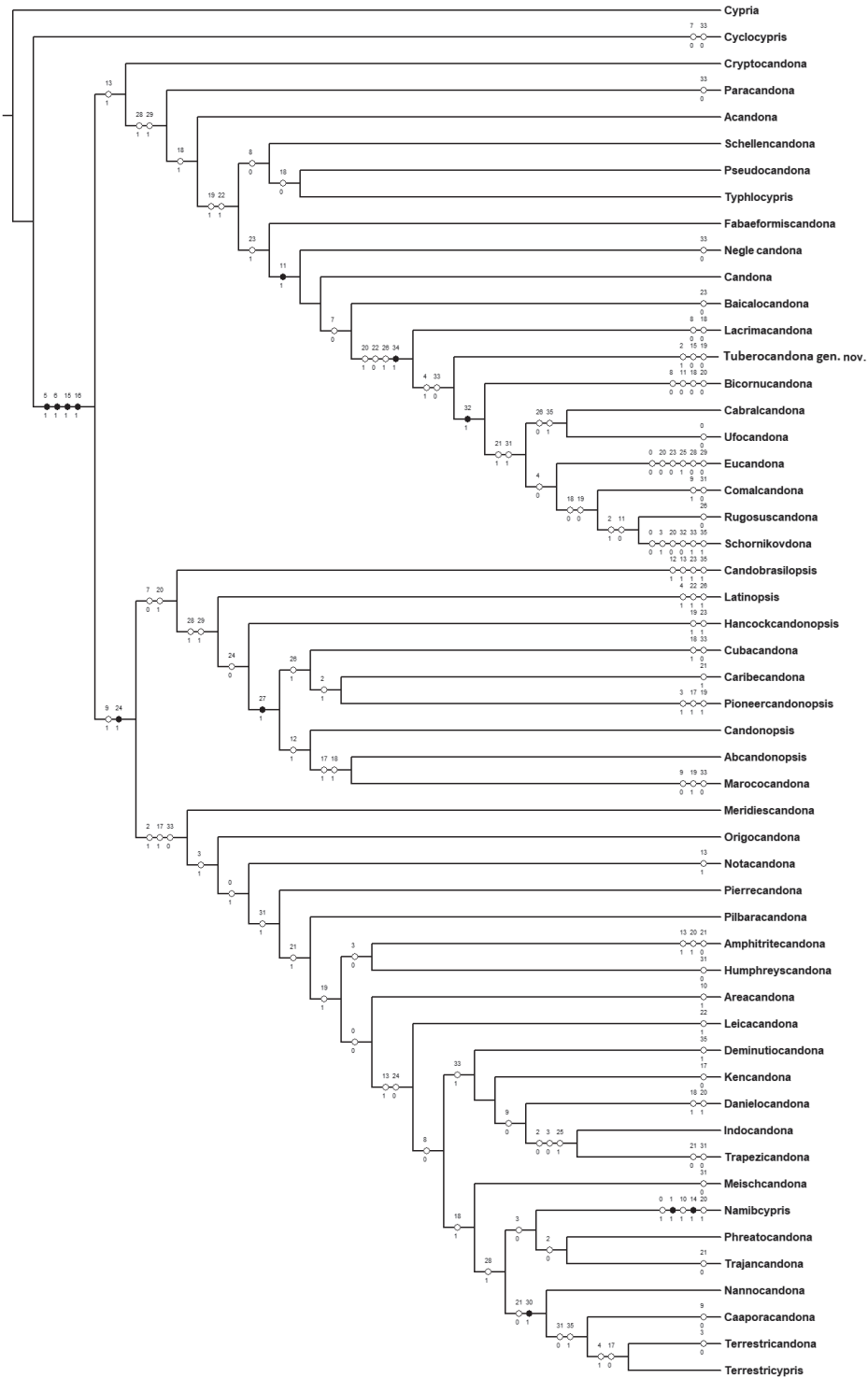
Figure 5. Clustering relationships among the 50 genera of Candoninae (plus two outgroup genera) including the new genus *Tuberocandona* gen. nov. See details in Appendix 1.

aperture of some pores is lobate, which is a character that is not known in any of the Candoninae members. Compared to the diameter of the pore, sensillum is very thin. Among the species of the tribe, *R. scharfi* has similar pore openings but much smaller and more abundant in numbers. Like the new species, *R. scharfi* was reported from groundwaters of the Edwards and Trinity associated aquifers.

Soft body parts and limb chaetotaxy

The new genus along with its type species has different and unique soft body parts and chaetotaxy in the limbs. The tribe Cabralcandonini covers species with five (*Schornikovdona bellensis*) (Külköylüoğlu et al. 2017d) to seven (e.g. *Lacrimacandona wisei*) ((Külköylüoğlu et al. 2017a) segmented A1. Having a 6-segmented A1, the new genus shows similarity to *R. scharfi*. Such reductions in some chaeototaxy of the soft body are known in some other species (Karanovic and Marmonier 2003; Higuti and Martens 2012; Smith and Kamiya 2015; Külköylüoğlu et al. 2017a, b, c, e). Therefore, those characters can illustrate derived character states (Danielopol 1980, 1982).

Sexual dimorphism in the A2 chaetotaxy is common in candonid species (see e.g. Meisch 2000; Karanovic 2007). This is also the case in the new genus (cf. Fig. 3B, E). For this reason, the lengths of the A2 claws are worth more discussion. *Tuberocandona* gen. nov. has long A2 claws which are almost equal or slightly longer than the length of all segments. Similarly, long claws illustrated in some other candonids (e.g. cf. *Ufocandona*) seem to be suitable for subterranean water conditions (Danielopol 1980). The exopodial plate of A2 carries one long and two very short setae both in males and females in the new genus (and species). This is similar in *C. tressleri*.

The t-setae (usually t2 and t3 setae) on A2 of many male candonids (e.g. *Schornikovdona*, *Lacrimacandona*) are transformed into a bristle-type that may be used during sexual courting. However, the t-setae are not transformed into bristles in the new genus like in *Rugosuscandona*, *Ufocandona* and *Comalcandona*. Although it is in a different tribe, similar reductions are also shown in *Indocandona rusti* (Külköylüoğlu et al. 2021), in which t-setae are bristle-type and the exopodial setae includes only two short setae which are barely seen at high magnification. The modifications in A2 mentioned herein may support an adaptive life to benthic subterranean aquatic conditions and/or habitats (e.g. springs) connected to subterranean water sources.

The Md of the new genus has a group of four smooth setae on distal end of segment 3l without alpha and beta setae. Except *Ufocandona*, absence of alpha and beta setae is not known in the tribe; all the species have a gamma seta with variations in length and shape. In the absence of an alpha seta, the new genus resembles *Rugosuscandona* and *Ufocandona*. In contrast, absence of the beta seta is only known in *Lacrimacandona*, *Schornikovdona* and *Ufocandona*. Terminal segment is fused with a thin and long claw in *Tuberocandona* gen. nov. similar to all other five genera discussed in here. Occurrence of a fused terminal claw is also known in members of different genera (e.g. *Phreatocandona*) (Danielopol 1973, 1982). In contrast, the fused terminal claw occurs only in females of *Trajancandona particular* (Karanovic 1999).

Presence of two smooth setae on the third endite of Mxl is also common character among the genera. However, there are sometimes differences between species.

According to the cladistic analyses (Fig. 5), differences in T1 structure were most effective for separating the new genus from others. Besides asymmetry in the male T1, not only the tribe Cabralcandonini but also many (if not all) other candonids appear to have distinguishing dissimilarities on T1. For example, right prehensile palp is very long in *U. hannaleeae*, robust and bumped shape in *S. bellensis*, and almost equal in *R. scharfi*. One of the common characters shared among the species of the tribe is the occurrence of the vibratory plate on T1. Except *R. scharfi*, all species have a single seta. Additionally, *Tuberocandona leonidasi* sp. nov. has two small a-setae when there is one a-seta in *Lacrimacandona* and *Comalcandona* but these setae are absent in *Ufocandona*, *Schornikovdona* and *Rugosuscandona*. Readers are advised to compare occurrence of b, c, and d setae of T1 among the species when differences may not be missed in the hemipenis of the males (Fig. 4E) and Zenker organ (Fig. 4F).

Tuberocandona leonidasi gen. nov. sp. nov. has one d-seta (d1) (e, d2, and dp are absent) on T2 and T3. Appearance of these setae show differences. For example, presence of d1 both in T2 and T3 is common among the species but e-seta of T3, except in *C. tressleri*, seem to be absent in all other species.

The uropod of *Tuberocandona leonidasi* gen. nov. sp. nov. has two well-developed claws and one very short but finger-like anterior seta (Fig. 4D). This structure is different from other species of the tribe. One common character observed in all the genera discussed here, however, is the absence of the posterior seta on the uropod. Thereby, absence of posterior seta is suggested for taxonomic classifications of the genera (and even for the tribe as well).

Conclusion

Based on the detailed morphological and cladistic comparative analyses described above, we conclude that *Tuberocandona* gen. nov. is a new genus of the tribe Cabralcandonini. Also, we report total of 673 ostracod fossil taxa in 142 genera found in Texas. However, we are aware that this number is not definitive and is likely to be increased by future studies. The Pennsylvanian period was the oldest period with 73 ostracod reports. Ostracod diversity (126 taxa) was the highest in the Eocene; however, a sharp decline in the numbers of taxa were seen after this period. It appears that only six species from the fossil record are currently extant. Including the new species described here, the numbers of non-marine living ostracods from the inland waters of Texas increased to 118 in 45 genera. Most of the nonmarine ostracods described from Texas in the last two decades are groundwater species. This trend is continued with the reporting of *Tuberocandona* gen. nov. sp. nov. herein, collected from a spring reliant on subterranean waters. This species decription contributed to the ever-growing knowledge of the groundwater diversity of Texas and emphasizes the need for further research and conservation efforts for these often rare and endemic species.

Acknowledgements

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Appendix I

Table A1. Total of 36 (0–35) morphological characters used in the cladistic analysis of the 49 genera belonging to nine tribes of the subfamily Candoninae (Karanovic 2007; Külköylüoğlu et al. 2021). Note to the two out group genera (*Cypria* and *Cyclocypris*) placed in the first two rows in the matrix. Character states: O.Surface of carapace: smooth / rarely ornamented (0), usually ornamented with hard ridges and/or holes (1); 1. Marginal pore canals: straight and equally long (0), branched and unequally long (1); 2. Number of Al segments: seven/eight (0), less (1); 3. Number of Al segments: 7/6 (0), 5 (1); 4. Exopod on A1: present (0), absent (1); 5. Rome's organ: present (0), absent (1); 6. Swimming setae on A2: present (0), absent (1); 7. Seta z1 on male A2: seta-like (0), claw-like (1); 8. Seta z2 on male A2: seta-like (0), claw-like (1); 9. G2 claw on female A2: shorter than Gl and G3 (0), equally long as Gl and G3 (1); 10. Number of rays on vibratory plate of Md: numerous (0), maximum of two (1); 11.Number of setae in bunch on Md palp: three (0), more (1); 12. Terminal segment on Md palp: square-shaped (0), several times longer than wide (1); 13. Setae in bunch on Md palp: wide and armed with only one row of setules (0), thin, and armed with many small setules all along and around (1); 14. Number of setae on protopod T1: numerous (0), only four (1); 15. Prehensile palps: segmented (0), unsegmented (1); 16. Prehensile palps: with additional subterminal bumps (0), without such bumps (1); 17. Basal seta on T2: present (0), absent (1); 18. Seta d2 on T3: present (0), absent (1); 19. Tf seta on T3: present (0), absent (1); 20. Posterior seta on CR: always present / very rarely absent (0), never present (1); 21. Posterior claw on CR: normal (0), reduced (1); 22. Appendage on genital field: never / extremely rarely present (0), always / most usually present (1); 23. Lobe g on hemipenis: very strongly sclerified (0), not strongly sclerified (1); 24. Lobe b on hemipenis: without chitinized dorsal part (0), with chitinized dorsal part (1); 25. Lobe a: normal (0), tiny and thin (1); 26. Lobe á: present (0), absent (1); 27. Lobe a: different (0), centrally positioned with lower and usually flat b and h lobes, or b lobe with a ventral projection (1); 28. Terminal segment of T3: with two short and one long setae (0), different (1); 29. Terminal segment of T3: different (0), with two long and one short setae (1); 30. Terminal segment of T3: different (0), Th2 seta transformed into long claw (1). 31. Exopod on A2: present (0), absent/reduced (1); 32. t setae on A2: present (0), absent (1); 33. Male sexual bristles on A2: absent (0); present (1). 34: Setae in bunch on Md palp: with row of setules (0), without setules (1). 35. Terminal segment of Mxl palp: normal (0); minute (1). Note that multiple and missing character states correspond to *, ?, respectively (adapted from Karanovic 2007, 2018) and characters (31–35) are newly used in this study. Empty and black circles represent alleged symplesiomorphic and alleged synapomorphic characters, respectively. Numbers below and above the branches show the code of the character state and character number, respectively. See details in Karanovic (2007).

	0	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	20	1	2	3	4	5	6	7	8	9	30	1	2	3	4	5
Tuberocandona gen. nov.	1	0	1	0	1	1	1	0	1	0	0	1	0	1	0	0	1	0	1	0	1	0	0	1	0	0	1	0	1	1	0	0	0	0	1	0
Cypria	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0
Cyclocypris	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Acandona	0	0	0	0	0	1	1	1	1	0	0	0	0	1	0	1	1	0	1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0
Amphitritecandona	1	0	1	0	0	1	1	1	1	1	0	0	0	1	0	1	1	1	0	1	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0
Areacandona	0	0	1	1	0	1	1	1	1	1	1	0	0	0	0	1	1	1	0	*	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0
Baicalocandona	1	0	0	0	0	1	1	0	1	0	0	1	0	1	0	0	1	1	0	?	1	0	1	0	0	0	0	0	1	1	0	0	0	*	0	?
Caaporacandona	0	0	1	1	0	1	1	?	?	0	0	0	0	1	0	?	?	1	1	1	0	0	0	?	?	?	?	?	1	0	1	0	0	0	0	1
Candona	0	0	0	0	0	1	1	1	1	0	0	1	0	1	0	1	1	0	1	1	0	0	1	1	0	0	0	0	1	1	0	0	0	1	0	0
Neglecandona	0	0	0	0	0	1	1	1	1	0	0	1	0	1	0	1	1	0	1	1	0	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0
Candonopsis	0	0	0	0	0	1	1	0	1	1	0	0	1	0	0	1	1	0	0	0	1	*	0	0	0	0	0	1	1	1	0	0	0	1	0	0
Abcandonopsis	0	0	0	0	0	1	1	0	1	1	0	0	1	0	0	1	1	1	1	*	1	*	0	0	0	0	0	1	1	1	0	0	0	1	0	0
Caribecandona	0	0	1	0	0	1	1	0	1	1	0	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	1	1	1	1	0	0	0	1	0	0
Cryptocandona	0	0	0	0	0	1	1	1	1	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	?

	0	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	20	1	2	3	4	5	6	7	8	9	30	1	2	3	4	5
Cubacandona	0	0	0	0	0	1	1	0	1	?	0	0	0	0	0	1	1	0	1	0	1	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0
Danielocandona	0	0	1	1	0	1	1	1	0	0	0	0	0	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0
Deminutiocandona	0	0	1	1	0	1	1	1	*	1	0	0	0	1	0	1	1	1	*	1	0	*	0	0	0	0	0	0	0	0	0	1	0	1	0	1
Eucandona	0	0	0	0	0	1	1	0	1	0	0	1	0	1	0	1	1	0	1	1	0	0	1	1	0	0	0	0	1	1	0	1	0	1	0	0
Fabaeformiscandona	0	0	0	0	0	1	1	1	1	0	0	0	0	1	0	1	1	0	1	1	0	0	1	1	0	0	0	0	1	1	0	0	0	1	0	0
Kencandona	0	0	1	1	0	1	1	1	0	1	0	0	0	1	0	1	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0
Humphreyscandona	1	0	1	0	0	1	1	1	1	1	0	0	0	0	0	1	1	1	0	*	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0
lndocandona	0	0	0	0	0	1	1	1	0	0	0	0	0	1	0	1	1	?	0	1	0	1	0	0	0	1	0	0	0	0	0	1	0	1	0	0
Leicacandona	0	0	1	1	0	1	1	1	1	1	0	0	0	1	0	1	1	1	*	1	0	*	1	0	0	0	0	0	0	0	0	1	0	0	0	0
Marococandona	0	0	*	*	0	1	1	0	1	0	0	1	0	0	1	1	1	1	1	1	1	*	0	0	0	0	0	1	1	1	0	0	0	0	0	0
Meischcandona	0	0	1	1	0	1	1	?	?	1	0	0	0	1	?	?	?	1	1	1	0	1	0	?	?	?	?	?	?	0	0	0	0	0	0	0
Meridiescandona	0	0	1	0	0	1	1	1	*	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Namibcypris	1	1	1	0	0	1	1	?	?	?	1	0	0	1	1	1	1	1	1	1	1	1	?	0	0	0	0	1	1	0	1	0	0	0	0	0
Nannocandona	0	0	1	1	0	1	1	?	?	1	0	0	0	1	0	?	?	1	1	1	0	0	0	?	?	?	?	?	1	1	1	1	0	0	0	0
Notacandona	1	0	1	1	0	1	1	1	*	1	0	0	0	1	0	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Origocandona	0	0	1	1	0	1	1	1	1	1	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Paracandona	0	0	0	0	0	1	1	?	?	?	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0	?	0	0	0	0	0
Phreatocandona	0	0	0	0	0	1	1	?	?	0	0	0	0	1	0	?	?	1	1	1	0	1	0	?	?	?	?	?	1	1	0	1	0	0	0	?
Pierrecandona	1	0	1	1	0	1	1	1	1	1	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0
Pilbaracandona	1	0	1	1	0	1	1	1	1	1	0	0	0	0	1	1	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0
Pioneercandonopsis	0	0	1	1	0	1	1	0	1	1	0	0	0	0	1	1	1	0	1	1	1	0	0	0	0	0	1	1	1	1	0	0	0	1	0	0
Schellencandona	0	0	0	0	0	1	1	1	0	0	0	0	0	1	0	1	1	0	1	1	0	0	1	0	0	0	0	0	1	1	0	0	0	1	0	0
Terrestricandona	0	0	1	0	1	1	1	?	?	1	0	0	0	1	0	?	?	0	1	1	0	0	?	?	?	?	?	?	1	0	1	0	0	0	0	?
Terrestricypris	0	0	1	1	1	1	1	?	?	1	0	0	0	1	0	?	?	0	1	1	0	0	?	?	?	?	?	?	1	0	1	0	0	0	0	1
Trajancandona	0	0	0	0	0	1	1	1	0	0	0	0	0	1	0	1	1	1	1	1	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0
Trapezicandona	0	0	0	0	0	1	1	1	0	0	0	0	0	1	0	1	1	1	0	*	0	0	0	0	0	1	0	0	0	0	0	0	0	*	0	0
Typhlocypris	0	0	0	0	0	1	1	1	0	0	0	0	0	1	0	1	1	0	0	1	0	0	1	0	0	0	0	0	1	1	0	0	0	1	0	0
Pseudocandona	0	0	0	0	0	1	1	1	*	0	0	0	0	1	0	1	1	0	0	1	0	0	1	0	0	0	0	0	1	1	0	0	0	1	0	0
Latinopsis	0	0	0	0	1	1	1	0	1	1	?	0	0	0	0	1	1	0	0	0	1	0	1	0	1	0	1	0	1	1	0	0	0	1	0	0
Rugosuscandona	1	0	1	0	0	1	1	*	*	0	0	0	0	*	0	1	1	0	0	0	1	1	0	1	0	0	0	0	1	1	0	1	1	0	1	0
Ufocandona	0	0	*	*	1	1	1	0	1	0	0	1	0	*	0	1	1	0	1	1	1	1	0	1	0	0	0	0	1	1	0	1	1	0	1	1
Cabralcandona	1	0	0	0	1	1	1	0	1	0	*	1	0	*	0	1	1	0	1	1	1	1	0	1	0	0	0	0	1	1	0	1	1	0	1	1
Lacrimacandona	0	0	0	0	0	1	1	0	0	0	0	1	0	*	0	1	1	0	0	1	1	0	0	1	0	0	1	0	1	1	0	0	0	1	1	0
Schornikovdona	0	0	1	1	0	1	1	1	*	0	0	0	0	*	0	1	1	0	0	0	0	1	0	1	0	0	1	0	1	1	0	1	0	1	1	1
Bicornucandona	1	0	0	0	1	1	1	0	0	0	0	0	0	*	0	1	1	0	0	1	0	0	0	1	0	0	1	0	1	1	0	0	1	0	1	0
Comalcandona	1	0	0	0	0	1	1	0	1	1	0	1	*	1	0	1	1	0	0	0	1	1	1	1	0	0	1	0	1	1	0	0	1	0	1	0
Hancockcandonopsis	0	0	0	0	0	1	1	0	1	1	*	0	0	0	0	1	1	0	0	1	1	0	0	1	0	0	0	0	1	1	0	0	0	1	0	0
Candobrasilopsis	0	0	0	0	0	1	1	0	1	1	0	0	1	1	0	1	1	0	0	0	1	0	?	1	1	0	0	?	0	0	0	0	0	1	0	1

RESEARCH ARTICLE

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How to map potential mesovoid shallow substratum (MSS) habitats? A case study in colluvial MSS

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Abstract

Understanding habitat extension that limits species distribution is a crucial tool for management and conservation, in which habitat mapping plays a pivotal role. The mesovoid shallow substratum (MSS) is a type of shallow subterranean habitat with an important conservation value for invertebrate communities, functioning as climatic/reproductive refuge, biogeographic corridor and/or permanent habitat. Methodologies to map the mesovoid shallow substratum (MSS) are currently lacking. We propose a novel method for colluvial MSS habitat mapping, combining geographic information systems, geological maps, and geological knowledge on the habitat genesis. We tested and validated the efficiency of the method using the Arrábida karst area (Portugal) as a model. The method allowed the remote detection of MSS habitats suitable for invertebrate communities *ex situ* within the study area, and enabled the estimation of habitat extent. The faunal communities sampled in the selected location were dominated by arthropods, especially insects, showcasing the efficacy of this mapping method to detect suitable MSS habitats. The use of this method considerably reduces the *in situ* scouting area, providing a more efficient way of locating these habitats. The MSS is protected under EU legislation concerning floral communities and geological features, completely neglecting its faunal communities. This method also allows to estimate potential MSS habitat extension in several lithologies, facilitating the implementation of invertebrate prospections, and the establishment of more effective conservation measures.

Introduction

The Mesovoid Shallow Substratum (or Stratum) was described in French as the "Milieu Souterrain Superficiel" (MSS) by Juberthie et al. (1980) in the Pyrenees, as an extensive type of shallow subterranean habitat, "formed by a network of air-filled voids and open spaces in between rock fragments" (for recent review on terminology see Mammola et al. 2016). Like caves, the MSS is characterized by total darkness, stable temperatures, and high humidity levels (Culver and Pipan 2014; Mammola et al. 2016; Nitzu et al. 2018).

Colluvial MSS, one of the four recognized types of MSS (Mammola et al. 2016), is typically composed of homogeneous rock material (depending on the type of bedrock) in various and heterogeneous sizes. The colluvial material consists of unconsolidated free sediments that have been deposited on hillslopes by either rain-wash, slow continuous downslope creep, or a combination of both these processes (Juberthie et al. 1980; Jiménez-Valverde et al. 2015). This type of MSS, when exposed is referred to as a scree slope, but can also be covered by a layer of soil and vegetation (Culver and Pipan 2009).

The MSS can be inhabited by organisms with different adaptations to life in subterranean habitats, from surface ones living in the soil (edaphic), to species with some affinities to the subterranean habitats (troglophile), to animals fully adapted and dependant on the subterranean habitat to survive (troglobiont) (Ortuño et al. 2013; Culver and Pipan 2014; Nitzu et al. 2014). It has been studied in several countries, mainly for its faunal communities, which are dominated by insects and other arthropods (Pipan et al. 2010; Rendoš et al. 2012; Ortuño et al. 2013; Nitzu et al. 2014; Jiménez-Valverde et al. 2015; Eusébio et al. 2021).

This habitat is an ecotone between surface and deeper subterranean habitats (Gers 1998), and can serve three very distinct, but equally important functions: 1) climatic/reproductive refuge for surface species, as in this habitat temperature variations along the year are much less extreme than at surface (Nitzu et al. 2011, 2014, 2018; Mammola et al. 2016); 2) biogeographic corridor to deeper ecosystems, as in many cases the MSS is connected to caves by a system of fissures, especially in karstic landscapes (Ortuño et al. 2013; Jiménez-Valverde et al. 2015); 3) permanent habitat for some species (Culver and Pipan 2009; Pipan et al. 2010; Eusébio et al. 2021).

Habitat mapping is a critical step for the management and conservation of habitats and their faunal communities (McDermid et al. 2005), as it allows an estimation of species distribution and richness patterns (Lecours et al. 2015), leading to the development of better monitoring protocols (Cogan et al. 2009; Lecours et al. 2015). Regarding subterranean habitats, improving habitat mapping methods will aid answering two

of the fundamental research questions on subterranean biology: "What is the species richness pattern of subterranean organisms globally?" and "What would be the best monitoring protocols to quantify long-term changes in the distribution and abundance of subterranean invertebrates?" (Mammola et al. 2020).

Subterranean habitats, "the most widespread non-marine environments on Earth" (Mammola et al. 2019), are particularly difficult to map due to their challenging access for humans (Mammola et al. 2019; Tanalgo et al. 2022). Caves have been mapped throughout the world since the beginning of speleological studies (Martel 1894), evolving into digital mapping (Trimmis 2018; Jones 2022; Kampolis et al. 2022), which include robots (Chang et al. 2022; Tabib et al. 2022) and remote sensing from space (Sharma and Srivastava 2022), using terrestrial lava tubes as models (Bell Jr. et al. 2022). On the other hand, the network of spaces and voids that composes the majority of subterranean habitats, which is inaccessible to humans, has never been mapped (Mammola et al. 2019).

This inaccessible underground network is the habitat that most subterranean species occupy, placing them "among the least documented fauna on our planet" (Mammola et al. 2019). Despite the different MSS types being properly described (Mammola et al. 2016), the worldwide extent of this habitat is currently unknown, with no available mapping methods proposed so far. Currently, its prospection relies solely on *in situ* visual verifications.

We propose a methodology to locate and estimate the areas of colluvial MSS habitats, using a combination of geographic information systems, geological maps, and geological knowledge on the genesis of this habitat. The Arrábida massif in Portugal was selected as the study area to develop, test, and validate the mapping method. The work conducted was divided into six steps: 1) defining a geological area to map, 2) defining main geological criteria for habitat genesis, 3) selecting potential locations using satellite imagery, 4) combining the potential sites with geological maps, in order to apply the criteria, 5) performing an *in situ* verification of the sites that met all the criteria, 6) assessing biodiversity in order to verify if the habitats selected by the method are suitable for typical MSS invertebrate communities.

Materials and methods

Literature review

Data on mapping methodology of shallow subterranean habitats was obtained from the available literature using: i) Elsevier Scopus with the following search topics "mesovoid AND shallow AND substratum" or "shallow AND subterranean AND habitats" or "milieu AND souterrain AND superficiel"; ii) Clarivate Web of Science with the same topics as i). Information harvested contains study location, MSS type, rock type, how the habitat was found, its extent, mapping method used, faunal taxonomic information, and type of results obtained in the study (Diversity, Taxonomy, Ecology).

Study area

The Arrábida karst massif (Portugal) was selected for the development of the habitat mapping method, due to its location in an area rich in invertebrate subterranean fauna (Reboleira et al. 2011), and the availability of geological maps and satellite images of the area.

The Arrábida chain is located in the southern edge of the Setúbal peninsula (Fonseca et al. 2014; Ramalho 2015), and is included in the homonymous Natural Park, created in 1976. The chain is particularly rich and diverse from a geological point of view (Parque Natural da Arrábida 2000), being comprised of a small group of mountains, oriented E-W and extending for 35 km, with the major accidents (thrusts and faults) verging to the South (Presas 2012; Fonseca et al. 2014), and a maximum altitude of around 500 m (Fonseca et al. 2015).

It is defined as a hard (high) relief, i.e., reliefs that stand out in height in comparison to the average altitudes of a region. This kind of hard reliefs are formed by the interaction of both erosive and tectonic processes (Fonseca et al. 2014). The erosive process occurs when there is a strong lithological contrast (Fonseca et al. 2014), i.e., when a relief has two different types of rock in contact with each other, one with a harder lithology (for example, crystalline limestones) and one with a softer lithology (for example, claystone or marlstone). In this case, there is a rapid erosion of the softer type of rock, leaving the harder one topographically exposed. This exposed hard rock will then erode slowly, first into boulders, and then into smaller and smaller rock fragments as they move down the slopes forming alluvial and colluvial fans. When rocky fragments reach the bottom of these slopes (and sometimes accumulate in deep structures) colluvial MSS is formed (Fonseca et al. 2015). The tectonic process consists of a compressive regime that resulted in the formation of faults and folds that sometimes duplicate by imbrication of the entire sequence. In the case of the Arrábida chain, these alternations oriented along the coast form cornices and cliffs, at the base of which the eroded sediments are deposited with little or no consolidation, forming colluvial MSS (Fonseca et al. 2014).

The Arrábida chain has a typical Mediterranean climate, characterized by two extreme seasons (a warm and dry summer, with prolonged drought periods, and a cold humid winter), and two mild seasons (spring and autumn) (ICNF 2015; Almeida et al. 2017). The fact that this chain faces the Atlantic Ocean leads to high air humidity levels (ICNF 2015). Its vegetation consists of a typical Mediterranean scrub, with *Ulex densus* and *Genista tournefortii* gorses, *Cistus albidus*, *C. ladanifer* and *C. monspeliensis* rockroses, *Quercus coccifera* and *Rhamnus alaternus* evergreen shrubs, and *Juniperus phoenicea* juniper (Parque Natural da Arrábida 2000).

Habitat mapping method

The first step in defining the mapping method for colluvial MSS habitats, of the scree slope type, was to define five main criteria for the selection of a location where this habitat might occur, taking into consideration all the conditions necessary for the geological formation of colluvial MSS (Table 1).

The second step was to select locations that seemed to possess "colluvial MSS properties", i.e., locations where slopes filled with rocky fragments could be detected visually, using Google Earth software.

The third step was to rasterize the geological map of the area, in this case section 38-B Setúbal, Portugal (Industry and Energy Ministry, Geology and Mining Institute) in QGiS 3.14.16 software (QGIS Development Team 2020), and select the following lithological features from the Jurassic and Cretaceous periods: limestone, conglomerate and dolomite. These three formation types were chosen according to the general lithology of the study area, and within the parameters of Criteria 4 (Table 1).

The fourth step was to overlap the coordinates of the selected potential colluvial MSS locations with the rasterized map in QGiS 3.14.16 software, using a mapping system that provides contour lines to better identify erosion run-off zones. Each location was tested on the resulting map for the fulfilment of all five criteria established for potential colluvial MSS habitat identification (Table 1).

The fifth step was to conduct an *in situ* verification for the locations which met all the five criteria. These locations were characterized for facing direction (registered *in situ*) and estimated area (calculated using Google Earth software) (Suppl. material 1).

The total estimated colluvial MSS area within the Arrábida Natural Park was calculated based on the individual estimated area of each scree slope.

Maps were produced in QGiS 3.14.16 software. An open access "World Topographic Map" layer (Esri 1995), which allows the identification of the erosion run-off zones at a 96 dpi resolution, was used in the details of Fig. 3 to provide contour lines.

Preliminary biodiversity assessment

A preliminary biodiversity assessment was performed following previous methodologies of measuring biodiversity in MSS habitats (López and Oromí 2010; Eusébio et al. 2021), installing five modified pitfall traps (Fig. 1: Location 15) at a depth of 50 – 60 cm during two months (February and March of 2022) in one of the sites detected as MSS habitats by this method. The collected specimens were identified to order level, using a Leica S6E stereomicroscope, were stored in 96% alcohol and deposited at the Faculty of Sciences and the National Museum of Natural History and Science, University of Lisbon, Portugal.

Results

Literature review

Methodologies to detect and map MSS habitat were not found in any of the papers surveyed, neither methodologies to estimate habitat extent (Suppl. material 2).

MSS habitats have been studied for diversity, taxonomy, and ecology of invertebrate fauna in 15 countries, where around 65% focus on colluvial MSS, and around 43% do not inform the reader about the lithology of the study area, being the dominant lithology (when mentioned) karst areas (around 39%) (Suppl. material 2).
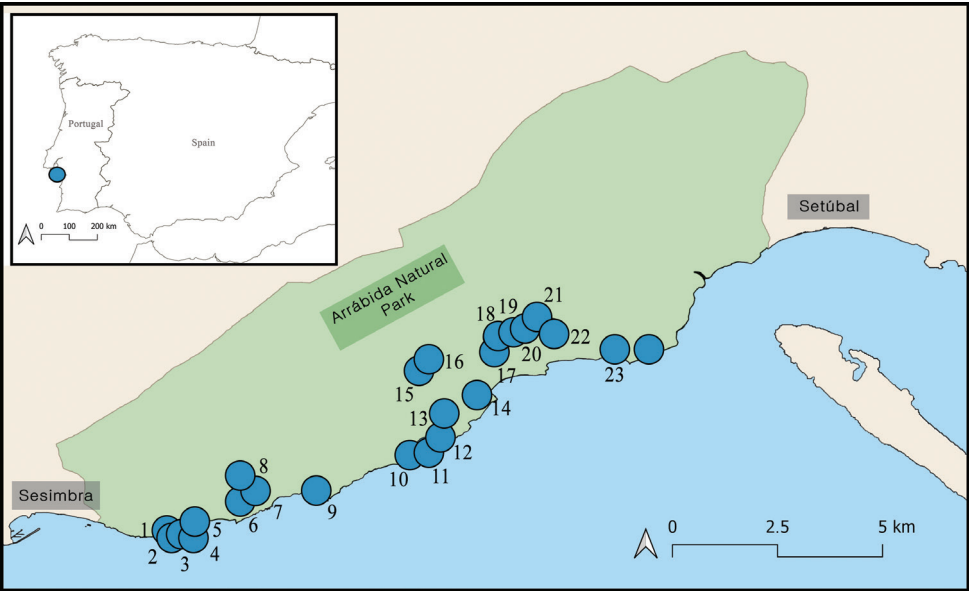
Figure 1. Potential colluvial MSS locations within the Arrábida Natural Park area, located between Sesimbra and Setúbal municipalities, Portugal.

Table 1. Criteria for identifying potential colluvial MSS habitats.

Criteria	Description	Justification
1	Reliefs present in the terrain	Reliefs consist of an exposed rock matrix that is elevated above ground level by either tectonic and/or erosive processes. This exposed matrix suffers subsequent erosion forming MSS habitats.
2	Strong lithological contrast in the relief	A strong lithological contrast means that there is a rapid erosion of a softer type of rock leaving adjacent harder rock exposed, leading to its later slow erosion into rock fragments and to the formation of scree slopes.
3	Erosion run-off zones	Considering that the parent rock is part of a relief, and that its erosion forms scree slopes, colluvial habitats form in steep slopes, below the exposed parent rock in the erosion run-off zone.
4	Adequate lithology	The type of rock selected needs to erode into fragments that can produce colluvial deposits. Example: limestones, conglomerates and marls (in karst areas), basalt (resultant of the erosion of volcanic cones).
5	Natural formation	The habitat needs to be formed by natural causes, and not by anthropogenic activities such as road construction and explosions associated with quarries, which form structures similar to colluvial MSS that are quite shallow or not connected with deep subterranean habitats.

Habitat mapping method

Potential colluvial MSS was initially indicated in 24 locations (Fig. 1). After applying the five criteria established for potential colluvial MSS habitat identification, 11 locations were considered eligible for an *in situ* verification (Suppl. material 3), of which eight were effectively confirmed to consist of scree slopes in the field (Figs 2, 3). The remaining three locations that fulfilled all criteria were observed in the field as completely covered by vegetation, and not as bare scree slopes.
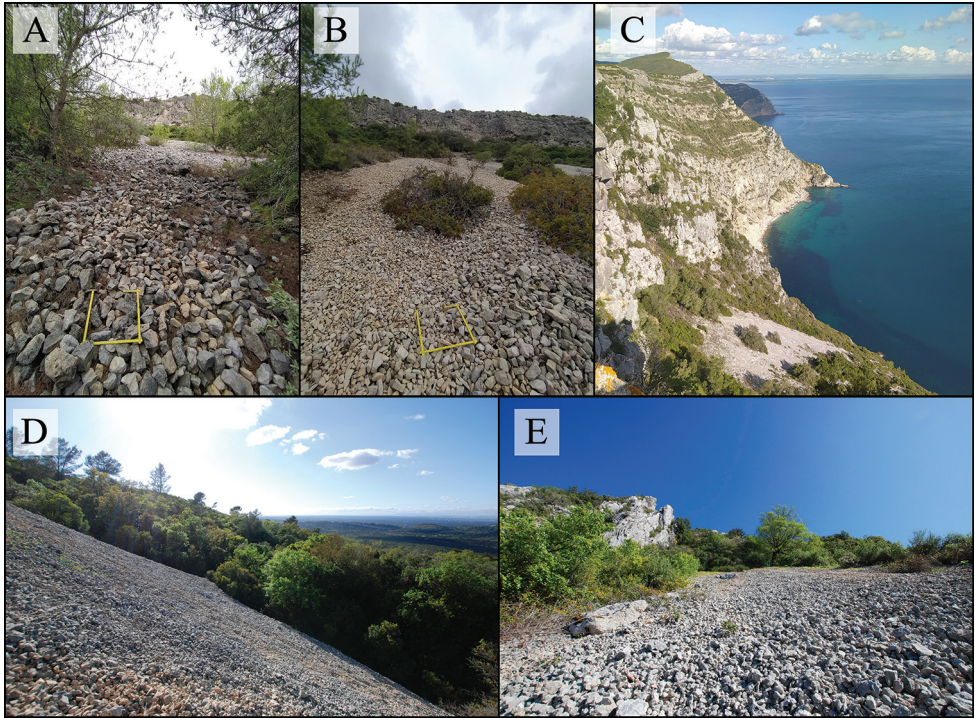
Figure 2. Locations that met all the five criteria established for potential colluvial MSS habitat identification. Locations: **A** 1 **B** 2 **C** 5 **D** 15 **E** 16.

Justification for locations meeting the defined criteria

Locations 1 through 4 are all within the erosion run-off zone right under 200 m a.s.l (Fig. 3, Table 1: Criteria 1, 3). Location 5 is located in the erosion run-off zone at an elevation of 280 m (Fig. 3, Table 1: Criteria 1, 3). Locations 1 through 3 consist of dolomitic rocky fragments, while locations 4 and 5 are crystalline limestone (Fig. 3, Table 1: Criteria 2, 4). All factors point to a natural formation of these scree slopes (Fig. 3, Table 1: Criteria 5) as they are situated in low human intervention areas, and are quite exposed facing south and southeast (Suppl. material 1) which in this case means that they face the sea being, therefore, very prone to erosion by strong winds and rain.

Locations 15 and 16 are also within the erosion run-off zone right under 300 m a.s.l., on a limestone area (Fig. 3, Table 1: Criteria 1–4). Again, these scree slopes are very likely formed by natural processes as there is a parent rock exposed to the elements which faces northwest (Suppl. material 1), in a pristine area (Table 1: Criteria 5).

Location 21 is situated within the erosion run-off zone at 240 m a.s.l., in a limestone area (Fig. 3, Table 1: Criteria 1–4). This scree slope is likely formed by natural processes as there is a parent rock exposed to the elements (Table 1: Criteria 5), which faces northeast (Suppl. material 1).
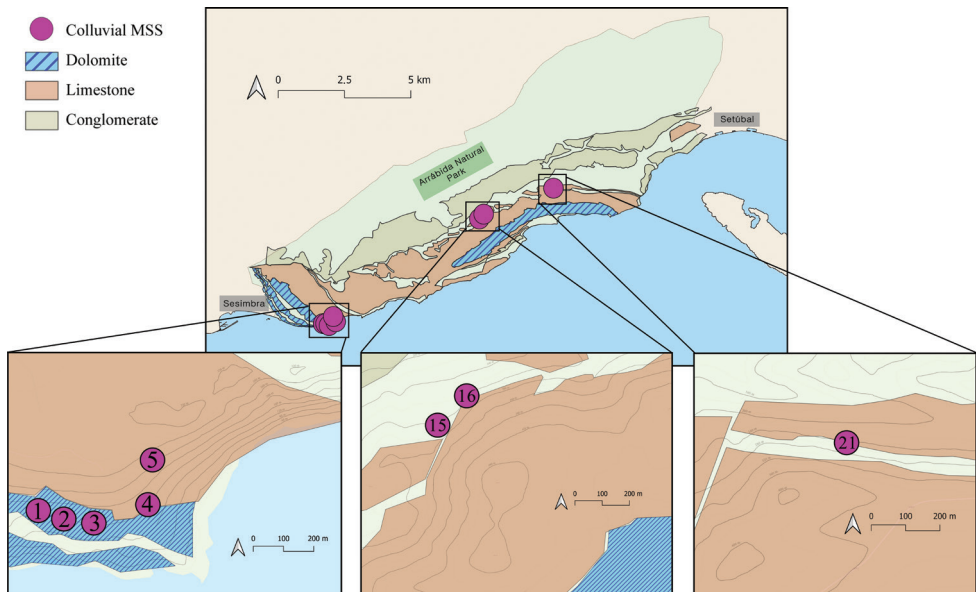
Figure 3. Map of the colluvial MSS locations confirmed *in situ*. Map of the Arrábida Natural Park, with the selected lithological feature layers (dolomite, limestone, and conglomerate), contour lines (in the three details), and colluvial MSS locations obtained with the proposed mapping method (numbered) confirmed *in situ*.

Estimated habitat area

The estimated areas for each of the scree slopes (Suppl. material 1) resulted in a total of approximately 30,500 m^2. This represents the total estimated surface area of colluvial MSS within the Arrábida Natural Park.

Habitat suitability for invertebrate fauna

A total of 551 invertebrate specimens were collected over the two winter months. About 98% of this community was represented by arthropods, with the remaining fauna being composed by molluscs (Suppl. materials 4, 5A). Over half of the arthropod specimens were insects, followed by Collembola (Suppl. materials 4, 5B). Within insects, Diptera's dominance was followed by Coleoptera and Hymenoptera (Suppl. materials 4, 5C). Among all taxa, a pseudoscorpion and a beetle presented troglomorphisms, such as reduced eyes and depigmentation (Suppl. material 4), but the lack of male specimens precluded the determination to species level.

Discussion

This new habitat mapping method, combining geographic information systems with geological maps and information on MSS genesis, allows tracking and mapping

colluvial MSS habitats, improving the current habitat location technique, which is based on *in situ* verification.

This method also allows for the estimation of habitat distribution, and when applied to faunal studies allows the estimation of species distribution areas. This knowledge will in turn contribute to improve the establishment of protection and conservation measures for this habitat and its faunal communities.

The application of this method requires: 1) deciding whether the formation of the scree slope is natural or anthropogenic, as this criteria can be biased; 2) selecting appropriate rock types within the lithology of the study site, as long as they allow the formation of scree slopes (this includes non-karst areas) and 3) taking into account vegetation coverage of the area, which can mask scree slopes in the *in situ* verification stage (Rendoš et al. 2014; Rudy et al. 2018; Jakšová et al. 2019). One method to overcome this last complication is to add an updated vegetation layer to the mapping step, when available, in order to better predict these events. Other approaches can involve long term surveys, as vegetation coverage varies over time, especially in areas prone to wildfires (Bastos et al. 2011).

The initial scan of the study area using satellite imagery resulted in 24 locations for potential MSS habitats. With the use of geographic information systems and geological maps this number was reduced to eleven. After the visual *in situ* verification, eight locations were confirmed as the intended habitat, i.e., bare scree slopes. Our method has, therefore, proven to be effective in locating potential colluvial MSS habitats with around 75% accuracy after the *in situ* verification, and also considerably reducing the scouting area in the field.

Our results suggest that the sole use of GIS software to locate MSS habitats is less effective than our mapping method. This can be attributed to the lack of satellite image updating on certain areas of the country, especially natural areas, where vegetation grows fast, drastically changing the landscape in a short time (Clemente et al. 1996; Meira-Neto et al. 2011). The discrepancy between the locations meeting all the criteria, and the ones detected in the field can be explained by vegetation cover. Therefore, the "missing" locations are in fact colluvial MSS habitats covered by the thick vegetation found all over the area of the natural park, mainly *Quercus coccifera* shrubs (Parque Natural da Arrábida 2000).

The *in situ* verification for the presence of colluvial MSS in these locations was prevented due to the extremely dense vegetation in the field, which would require cutting down natural vegetation patches. MSS covered with soil and vegetation is common in the margins of bare scree slopes, which are gradually covered by mature soils, leading to more extensive plant coverage over time (Mammola et al. 2016; Giupponi et al. 2023). Therefore, our method also has the potential to detect "hidden" MSS areas, which are covered by soil and vegetation, continuous to the detected bare scree slopes.

In fact, the lithology of the area, previous observations, especially historical events related to wildfires (Clemente et al. 1996; Cruz and Viegas 1997), and knowledge of the natural park landscape (Parque Natural da Arrábida 2000) indicate the presence of MSS covered by soil and vegetation in those "missing" locations.

The mapping method also proved to be efficient in locating habitats suitable for invertebrate fauna. Scree slope habitats can host both surface and subterranean-adapted species (Pipan et al 2010; Nitzu et al. 2011, 2014; Jiménez-Valverde et al. 2015). The communities found in the sampled location have a similar composition to communities typically found in the MSS in other European countries, i.e., the absolute dominance of arthropods, more specifically insects (Rendoš et al. 2012; Ortuño et al. 2013; Jiménez-Valverde et al. 2015; Eusébio et al. 2021), and few troglobiont species (Pipan et al. 2010; Jiménez-Valverde et al. 2015). However, we found an unusual pattern, the clear dominance in number of individuals of Diptera over Collembola. This can be linked with the lower altitude of our study (300 m a.sl.), in contrast with the high mountain location of the previous studies (Rendoš et al. 2012; Jiménez-Valverde et al. 2015), as the abundance of several Diptera are known to be affected by elevational gradients and other climatic variables (Hodkinson 2004; Rhoner et al. 2015), but also by the lack of soil and vegetation coverage of bare scree slopes, that may facilitate the colonization of this habitat by flying insects.

This method allows a habitat extent estimation, which in the case of the colluvial MSS within the Arrábida Natural Park is approximately 30,500 m². This value corresponds to the surface area of habitat. However, like in other habitats, such as coral reefs (Dustan et al. 2013), the real extension of the MSS habitat is higher than its surface area, due to its three-dimensionality. Future studies to quantify the total MSS 3D area and volume will have to consider habitat depth and slope, rock fragment size, and rock occupied volume versus air filled spaces. It is also important to consider the area of the habitat that is covered by vegetation.

In Portugal, MSS habitats are protected by EU legislation in the Habitat Directive as "Western Mediterranean and thermophilic deposits", in the "Limestone scree slopes (8130pt1)" subcategory, regarding plant communities and geological features (ICNB 2000). However, this protection completely disregards faunal communities, and, in a recent study, the Portuguese MSS was considered a habitat with high conservation value for invertebrate communities (Eusébio et al. 2021).

The use of this mapping method might facilitate the detection of MSS, which can later be studied and contribute to overcome the main shortfalls of subterranean biodiversity (Mammola et al. 2019) in shallow subterranean habitats. It will enable the increase of knowledge about habitat distribution (Racovitzan shortfall), which will facilitate new faunal community studies (Mammola et al. 2019).

This new mapping method has the potential to be used worldwide, paying special attention to integrate topography, geomorphology, lithology and tectonic structures of the studied location, improving work efficiency for studies that deal with colluvial MSS, a more and more important habitat in the context of global changes as a climatic/reproductive refuge (Eusébio et al. 2021).

The possibility to survey the area of shallow subterranean habitats worldwide will help define conservation perimeters, and estimate the extent of occurrence (EOO) and area of occupancy (AOO) according to the IUCN Red List criteria (IUCN 2021) for species that inhabit shallow subterranean habitats. Moreover, our method allows for the estimation of the extension of this type of habitat around the world, which will certainly contribute to establish more effective conservation measures for land-use at surface and for faunal conservation.

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Supplementary material 1

Characterization of each of the locations found in situ as colluvial Mesovoid Shallow Substratum (MSS): latitude, longitude and estimated area (m²)

Authors: Rita P. Eusébio, Paulo E. Fonseca, Rui Rebelo, Maria da Luz Mathias, Ana Sofia P. S. Reboleira

Data type: table (Excel file)

Link: https://doi.org/10.3897/subtbiol.45.96332.suppl1

Supplementary material 2

Literature review data Mesovoid Shallow Substrate's faunal communities

Authors: Rita P. Eusébio, Paulo E. Fonseca, Rui Rebelo, Maria da Luz Mathias, Ana Sofia P. S. Reboleira

Data type: Excel file

Link: https://doi.org/10.3897/subtbiol.45.96332.suppl2

Supplementary material 3

Results of criteria met for each location pinpointed as potential colluvial Mesovoid Shallow Substratum (MSS)

Authors: Rita P. Eusébio, Paulo E. Fonseca, Rui Rebelo, Maria da Luz Mathias, Ana Sofia P. S. Reboleira

Data type: Excel file

Link: https://doi.org/10.3897/subtbiol.45.96332.suppl3

Supplementary material 4

Abundance of invertebrates collected in colluvial Mesovoid Shallow Substratum (MSS) at the Arrábida National Park

Authors: Rita P. Eusébio, Paulo E. Fonseca, Rui Rebelo, Maria da Luz Mathias, Ana Sofia P. S. Reboleira

Data type: Excel file

Link: https://doi.org/10.3897/subtbiol.45.96332.suppl4

Supplementary material 5

Total invertebrate abundance, collected in colluvial Mesovoid Shallow Substratum (MSS) at the Arrábida National Park

Authors: Rita P. Eusébio, Paulo E. Fonseca, Rui Rebelo, Maria da Luz Mathias, Ana Sofia P. S. Reboleira

Data type: figure (.tiff file)

Explanation note: **A.** Total abundance of arthropods and molluscs; **B.** Total abundance of Arthropoda classes; and **C.** Total abundance of Insecta orders.

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Link: https://doi.org/10.3897/subtbiol.45.96332.suppl5

SHORT COMMUNICATION

Evidence for metapopulation structure in seep-dwelling amphipods

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Abstract

Twelve seepage springs in a 3 km² area of the Goldmine Tract in the C&O Canal National Historical Park in Montgomery County, Maryland, USA were sampled in 1994–1995 and again in 2023 for three amphipod species: *Stygobromus tenuis potomacus*, *Stygobromus pizzinii*, and *Crangonyx shoemakeri*. During that time interval, there were 11 colonizations and 1 exinction. Three populations persisted. These results are consistent with the proposed metapopulation hypothesis of population structure.

Keywords

Hypotelminorheic, springs, stygobionts, subterranean, urban conservation

Introduction

The most superficial shallow subterranean aquatic habitat, the hypotelminorheic, first described in the Medvednica Mountain of Croatia by Meštrov (1962), remains enigmatic. As described by Meštrov (1962, 1964), and later elaborated by Culver et al. (2006) and Pipan et al. (2012), it is a miniaturized subterranean drainage basin, underlain by clay, with a surface projection of about one hectare (see Staley 2016). The depth of the clay layer from the surface varies, but typically is a meter or less. The surface

manifestation of the hypotelminorheic is a seep, or seepage spring. Seepage springs are usually little more than wet spots in the woods. They typically have low flow that may not be discernible, occur in a slight depression, and have blackened leaves. During the summer months, the seeps are generally dry, with the only water being that in the colloidal clays. Although similar habitats have been reported in grasslands and even in tile drains of cultivated fields (Koenemann and Holsinger 2001; Culver et al. 2012), most hypotelminorheic habitats are in forested areas.

Hypotelminorheic habitats have proved very difficult to sample and to date, only by direct examination of the leaves and sediment of the seepage spring (Culver et al. 2006) or eDNA analysis of water samples has proven effective (Niemiller et al. 2017). Furthermore, seepage springs can only be sampled from late fall (leaf fall) to early spring (leaf out). During the rest of the year, presumably most of the superficial groundwater is taken up by evapotranspiration.

In the Potomac River basin of eastern USA, the region from Washington, DC to Cumberland, MD (approximately 160 km) has been intensively studied (e.g., Feller 1997; Hobson 1997; Culver and Šereg 2004) for the past several decades. From this work, a number of conclusions and working hypotheses have been formulated, listed in order of decreasing certainty. First, the habitat harbors a rich fauna specialized for subterranean life. There are amphipod, isopod, and snail species, with reduced or absent pigment and eyes. The highlight of this assemblage in the Potomac River basin around Washington, DC are the six species of blind, depigmented amphipods in the genus *Stygobromus* (Pipan et al. 2012). Except for *S. pizzinii*, which is found in a few caves in Pennsylvania, none of the species are found in either surface habitats or caves. Second, hypotelminorheic habitats tend to be clustered, presumably where a superficial clay layer is present. Where they are present, they form a mosaic of polygons defined by the movement of shallow subsurface water. Third, the seeps are more than exit points where hypotelminorheic animals get washed out. Currents are too low and animals are too large for the presence of animals at seeps to be accidental. It is possible that occasional currents are strong enough to dislodge amphipods from seeps, but it is certainly rare at best. Fong (unpublished but summarized in Culver and Pipan [2014]) shows that the presence of *Stygobromus tenuis potomacus* in a seep near Pimmit Run in the George Washington Memorial Parkway, Virginia, is highly temperature dependent, and they are nearly absent at higher temperatures occurring in the summer. This suggests that they are foraging for food in the relatively resource rich seepage spring. Fourth, habitat quality and the likelihood of finding species specialized for seeps is correlated with canopy height and presumably the quality of the leaf litter in the habitat (Burch et al. 2022). Fifth, the curious finding that no basic physico-chemical factor was a good predictor of the presence of animals, suggested that these were metapopulations, where individual populations blink on and off through time (Keany et al. 2018). However, individual species showed significantly different physico-chemical niches, particularly with respect to temperature and conductivity.

Metapopulation dynamics, first elaborated by Levins (1969) and much elaborated since (Hanski and Simberloff 2007), proposes that populations be governed by the balance between immigration and extinction, as well as the balance between birth and

death rates of local subpopulations. A metapopulation structure for hypotelminorheic species is highly conjectural because of the lack of direct evidence, especially evidence that sub-populations blink on and off.

The purpose of this note is to provide evidence for population extinction and colonization in a series of hypotelminorheic habitats in the C&O Canal National Historical Park in Montgomery County, Maryland, USA, based on samples of the same seeps in 1994–5 and 2023, a period of 28–29 years.

Methods

In 1996, Feller (1997) sampled 12 seeps in an area of 1500 by 2100 m in the Gold Mine Tract (Fig. 1) near the Mather Gorge section of the Potomac River near Washington, D.C. This is a mature hardwood forest dominated by oaks (*Quercus*), poplar (*Populus*), and beech (*Fagus*), with abundant foot trails, both official and "social." The area is bisected by the Gold Mine Loop Trail. The water coming from the seeps coursed through a thick leaf and woody detritus in natural channels, terminating in the C&O Canal. The geology of the site is mapped as schist-Wissahickon Formation, Marburg Schist. Fine grained mica was commonly observed in seep runs. A typical seep (#11) is shown in Fig. 2.

In 2023, all 12 seeps were relocated and resampled for the following amphipod species in the family Crangonyctidae:

- *Stygobromus pizzinii* (Shoemaker, 1938)
- *Stygobromus tenuis potomacus* (Holsinger, 1967)
- *Crangonyx shoemakeri* (Hubricht & Mackin, 1940)

S. pizzinii and *S. tenuis potomacus* are without eyes or pigment, and except for a handful of caves in Pennsylvania where *S. pizzinii* has been found, they are limited to seeps. *C. shoemakeri* is common in seeps but also occurs in wetlands (Zhang and Holsinger 2003; Keany et al. 2018).

Collections in 1994–5 and 2023 were made in the same way–the hand examination of the litter and gravels at the exit of the hypotelminorheic–the seep. Each seep was sampled for a minimum of 30 person minutes. The 1994–1995 samples were taken in March and April, and the 2023 samples were taken in February, all times of relatively cold water and well before leaf out.

Results

The results of the census are listed in Table 1. During the 2023 census, an additional seep was found, near seep #9. This new seep had *S. tenuis potomacus*.

Between 1996 and 2023, *S. pizzinii* colonized two seeps, and persisted in one. *S. tenuis potomacus* colonized two seeps, went extinct (or at least were undetectable)
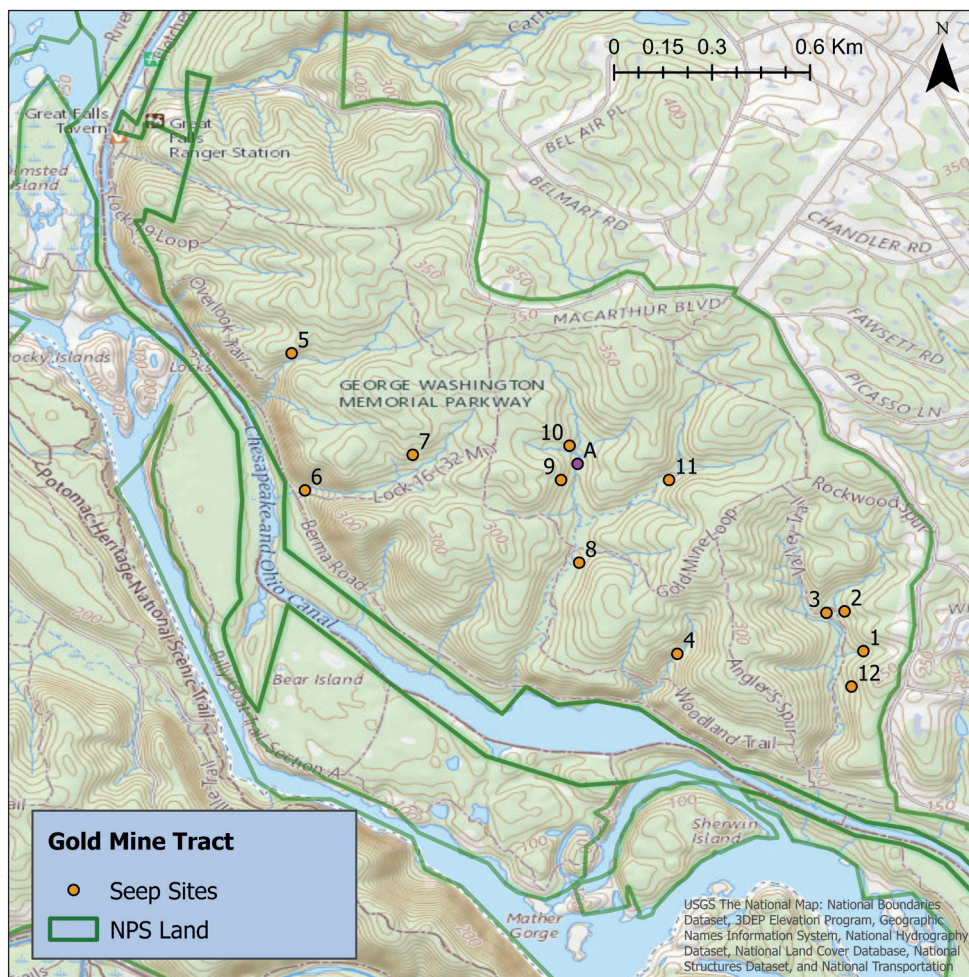
Figure 1. Map of study site. Numbers are from Feller (1997). The letter A indicates the location of a new seep discovered in 2023.

in one, and persisted in one. *C. shoemakeri* colonized six seeps and persisted in one. The one "new" seep (A in Fig. 1) had a population of *S. tenuis potomacus*. The major changes in 2023 was the occurrence of the six "new" seeps with *C. shoemakeri* and an increase in the number of seeps with detectable fauna by four.

Discussion

Our working hypothesis is that the seep dwelling amphipod populations form a metapopulation, as depicted schematically in Fig. 3. We have documented the changes that have occurred over a 25+ year period, and these include colonizations and extinctions, with more colonizations than extinctions (Table 1). While it is pos-

Figure 2. Seep 11 (see Fig. 1). Skunk cabbage (*Symplocarpus foetidus*) is growing around the seep, a frequent occurrence.

Table 1. Species composition of seeps in the study area (see Fig. 1) in 1994–5 and 2023. Numbers of individuals collected in 2023 are given in parentheses.

Seep No.	1994–5	2023
1	*S. pizzinii*	*S. pizzinii* (5), *S. tenuis potomacus* (3), *C. shoemakeri* (1)
2		
3		*S. tenuis potomacus* (2), *C. shoemakeri* (4)
4		*C. shoemakeri* (6)
5	*C. shoemakerii*	*S. pizzinii* (1), *C. shoemakeri* (4)
6		
7		
8	*S. tenuis potomacus*	*S. tenuis potomacus* (3), *C. shoemakeri* (6)
9		
10		*C. shoemakeri* (2)
11		*C. shoemakeri* (5)
12	*S. tenuis potomacus*	*S. pizzinii* (17)
A		*S. tenuis potomacus* (5)

sible that some populations were not detected during both censuses, it is also possible that more extinctions and recolonizations occurred when, for example, an extinction was followed by a recolonization during the rather long time period between sam-
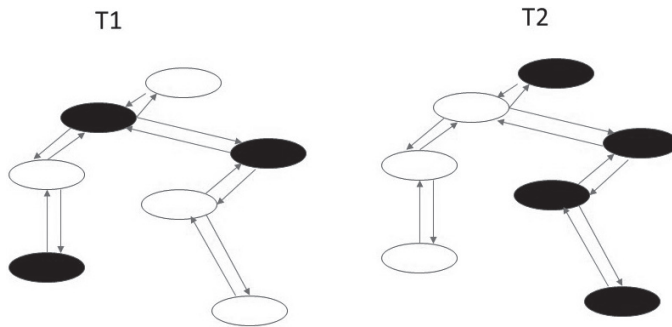
Figure 3. Representation of a metapopulation at two points in time. Black ellipses represent occupied seeps, white ellipses unoccupied seeps. Arrows are possible dispersal paths.

plings. Nonetheless, we believe the sampling resulted in the detection of many if not all populations.

The discovery of an additional seep in 2023 may be because it was not present in 1996. The hypotelminorheic habitat and the accompanying seep are transitory habitats, which exist almost exclusively in shallow soil and leaf litter. Minor alterations of the landscape, such as soil compaction, can change the location of seeps.

An especially pernicious problem in the study of the hypotelminorheic is the problem of false negatives. With the exception of eDNA, no technique has been found that is reliable as direct hand sampling (see Culver and Šereg 2004), and the success of hand sampling is somewhat dependent on flow rates of seeps. Flow rates in seeps have a complex relationship with precipitation since most precipitation is taken up by plant transpiration during the warmer months. Winter and spring precipitation (https://www.weather.gov/media/lwx/climate/dcaprecip.pdf), the time of maximum seep flow, was highest in 1994 (62.1 cm), lowest in 1995 (39.4 cm), and intermediate in 2022 and 2023 (49.8 cm), suggesting the changes we observed were not based on rainfall. A more accurate assessment would be to do repeated daily or weekly sampling, but this would be destructive to the habitat. Niemiller et al. (2017) demonstration of the feasibility of eDNA analysis to determine presence or absence of amphipods in seeps offers a way forward and an improvement on the present study. The only caveat is that the persistence time of eDNA in such subterranean habitats in general is unknown (Boulton et al. 2023). Finally, we note that our sample size was small (n = 12), not a statistically sufficient number for analysis. In spite of these caveats, we think the metapopulation hypothesis is worth pursuing.

A key remaining question is how migration and recolonization occur. Fong (quoted in Culver and Pipan [2014]) demonstrated that in a seep along Pimmitt Run in the George Washington Memorial Parkway in Virginia, numbers of *S. tenuis potomacus* increased with decreasing temperature. This result implies that with cooler temperatures, the amphipods are more active foraging in the seep. Dispersal may occur when conditions are wet (and cool) enough. Water, usually storm runoff, in a thin layer over the ground surface, may be especially important. Sheet flow provides a temporary dispersal corridor both for active and passive dispersal.

While dispersal via groundwater is possible, groundwater is typically many meters below the hypotelminorheic. At least *S. tenuis potomacus* and *C. shoemakeri* can survive periods of drying by burrowing into the clay layer (Gilbert et al. 2018) that typically lines the hypotelminorheic habitat (Staley 2016) a meter or so below the surface.

Finally, we note that the metapopulation model has profound implications for conservation planning. Rather than focus on the above ground aquatic habitat, especially feeder and base-level streams, the focus should be on the forest litter and its health and connectivity. It is not just the seeps occupied at one point in time that need protection, but also unoccupied seeps, that may be occupied later, and the dispersal paths between hypotelminorheic habitats (Fig. 2). The forest litter is not only important for dispersal – it appears that habitat quality is positively correlated with canopy maturity and height (Burch et al. 2022). The metapopulation and its dispersal paths are present in the shallow subsurface–the leaf litter, the hypoelminorheic, and seepage springs.

Conclusions

The biology of the amphipod and isopod species remains largely enigmatic. Their habitat is the most superficial of subterranean habitats, yet many of its inhabitants show strong features of adaptation to aphotic environments, including loss of eyes and pigment. This study strengthens the hypothesis that seeps represent semi-isolated populations that blink on and off through time. Their persistence depends not only on occupied seeps but also on unoccupied seeps and a means of dispersal between them.

Acknowledgements

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RESEARCH ARTICLE

The overview of lithobiomorph centipedes (Chilopoda, Lithobiomorpha) from caves of Slovenia

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Abstract

Centipedes of temperate regions can be found in various habitats, including forest litter, soil or caves. Slovenia, situated in the Northwestern Balkans, has rich centipede fauna, with one of the earliest scientific descriptions of a cave centipede, *Lithobius stygius* Latzel, 1880, from Postojnska jama. Many lithobiomorph species have been reported from Slovenian caves, but the data on their occurrence are scattered in the literature or public collections, and several specimens have even remained unexamined. Here we present the overview of lithobiomorph centipedes found in Slovenian caves. Altogether, 21 lithobiomorph species were found in 160 localities. The majority of the records, 319 out of 410, are published for the first time. Only three species are considered exclusively subterranean species, *L. stygius*, *L. zveri* and *Eupolybothrus obrovensis*, while other species are surface dwellers. The potential explanation of surface species presence in caves is discussed. We comment on cases of unresolved taxonomical status and present suggestions for further research needed to resolve them. Even though lithobiomorph centipedes often occur in caves, their role and importance in subterranean habitats remain to be studied.

Keywords

Introduction

Centipedes are important predators in various ecosystems, ranging from tropical to subpolar climates (Voigtländer 2011). In temperate regions, where they reach very high species richness, centipedes inhabit various habitats, including forest litter, soil, human buildings and caves, with humidity being the crucial factor influencing their occurrence (Bonato and Zapparoli 2011; Voigtländer 2011). Even though centipedes have regularly been found in caves all over the world, only a small share of them are presumed to be troglobitic (e.g., Edgecombe 2005; Chagas-Jr and Bichuette 2018; Edgecombe et al. 2020; Stojanović et al. 2021). The interest for the centipedes in caves has a rich history where the first subterranean centipedes were described in France (e.g., *Lithobius coquerellii* Lucas, 1860, *Lithobius cavernicolus* Fanzago, 1877 and *Lithobius speluncarum* Fanzago, 1877), but reports on their occurrence date even earlier. In the Balkans, the first described subterranean centipede was *Lithobius stygius* Latzel, 1880 from Postojnska jama, in Slovenia (Latzel 1880). Most of the subterranean species belong to the order Lithobiomorpha, however, some troglobiotic Geophilomorpha and Scolopendromorpha have been described as well, also from the Balkans (Stoev et al. 2015; Shear and Krejca 2019; Vahtera et al. 2020).

With more than 250 species, the Balkan Peninsula is one of the richest regions in the world regarding the centipede species richness (Bonato and Zapparoli 2011; Simaiakis and Strona 2015), of which more than half (142 species) belong to the order Lithobiomorpha (Stoev 1997). At its Northwestern part, in Slovenia, there is relatively high proportion of centipede species richness – between 58 (Bonato et al. 2016) and 86 (Stoev 1997) species are reported in the checklists, around half of them belonging to the order Lithobiomorpha, family Lithobiidae (Kos 1992a; Stoev 1997; Simaiakis and Strona 2015; Bonato et al. 2016). Three lithobiid genera were reported for the country, namely *Lithobius* Leach, 1814, *Harpolithobius* Verhoeff, 1904 and *Eupolybothrus* Verhoeff, 1907.

The knowledge on the centipede diversity on the area of today's Slovenia, including caves, accumulated over time. The first important contributions were by Latzel (1880); Verhoeff (1929, 1930, 1933, 1937b, 1943); Attems (1908, 1929, 1959) and Manfredi (1932b). In the second half of the 20th century, centipedes gathered in caves were studied mostly by Matic (Matic and Dărăbanţu 1968; Matic and Stentzer 1977; Matic 1978, 1979). Their work resulted in the description of three currently valid exclusively subterranean species, *Lithobius stygius* Latzel, 1880, *Lithobius zveri* (Matic & Stenzer, 1977) and *Eupolybothrus obrovensis* (Verhoeff, 1930). More recently, the data on centipedes in caves was gathered sporadically in general overviews of centipedes diversity (e.g. Zapparoli 1989; Kos 1992a) or during cave fauna inventories (e.g. Novak 2005; Polak et al. 2012; Polak and Pipan 2021; Zagmajster et al. 2021). In the last two decades, a lot of new centipede material from caves has been collected as part of different faunistic studies. It mostly remained as grey literature (reports, bachelor thesis), unpublished or even as unexamined material. Furthermore, the data was scattered in various literature sources and several collections and there was no overview of species found in caves of the country.

To fill the gap and present the up-to-date overview of the lithobiomorph centipedes in the caves of Slovenia, we have compiled the available data from published and unpublished sources. Besides giving the overview of the records and the species list, we

present taxonomic challenges regarding the identification of species and recommend the main directions for further studies on this important, yet heavily understudied group.

Materials and methods

Geographical setting

Slovenia, with an area of 20,273 km², lies at the junction of four major European geographical macro-regions: the south-eastern part of the Alps, the western margin of the Pannonian Basin, the north-western part of the Dinaric Alps and the northern margin of the Mediterranean (Perko et al. 2020). The country's complex geological history has influenced the development of various landforms, topography, bedrock, soil types, hydrology, climate and vegetation. The climate ranges from sub-Mediterranean to temperate continental and montane (Perko et al. 2020). On average, there is 1750 mm of precipitation annually, which are spatially and temporally highly variable (Komac et al. 2020). The average annual temperature in Slovenia is between 10 and 13 °C (Komac et al. 2020). The primary vegetation in most parts of Slovenia is temperate forest (Andrič and Willis 2003; Šilc et al. 2020), which covered 58% of the country in 2020 (SURS 2021).

An important geographical feature of Slovenia is the karst, special type of landscape, where water soluble carbonate bedrock enables formation of fissures and caves (Gams 2004). There are over 14,000 caves registered in Slovenia, some of which are more than 1000 m deep (Karst Research Institute ZRC SAZU 2022; Ljubljana Cave Exploration Society 2022). Karst areas cover 43% of the country and are divided into three principal areas: Alpine karst, Isolated karst and Dinaric karst (Gams 2004; Zupan Hajna 2004).

Data compilation

Information on the occurrence of lithobiomorph species in caves was taken primarily from the database on subterranean biodiversity SubBioDB (managed by the Subterranean Biology Lab, Department of Biology, Biotechnical Faculty, University of Ljubljana), including data until the end of 2022. SubBioDB combines literature data and the data on individuals collected during various fieldwork expeditions. One record refers to the unique combination species-locality-source for literature data and species-locality-date for new (fieldwork) data.

Specimens reported as new findings were collected in the fieldwork by visual inspections and direct hand picking or, in some cases, by baited pit-fall traps. All individuals were preserved in 70% or 96% ethanol and stored in the Zoological collection of the Subterranean Biology Lab, Department of Biology, Biotechnical Faculty, University of Ljubljana. Some specimens were contributed to this collection from the collections of the Notranjska Museum in Postojna (Slavko Polak), or the speleobiological collection of the Department of Biology, Faculty of Natural Sciences and Mathematics, University of Maribor (Peter Kozel). In addition, some older material was taken from the "Chilobio" centipede collection of the Animal Ecology Group, University of Ljubljana (Ravnjak

and Kos 2015). Specimens were examined under stereomicroscope Olympus SZH10 or Leica M165 C and morphologically determined using identification keys for lithobiomorph centipedes (e.g. Matic 1966; Koren 1992; Stoev et al. 2010). In cases where reliable species identification was not possible, e.g., due to damaged, juvenile or female individuals, specimens were identified only to a genus level.

Species taxonomy followed the one proposed in ChiloBase 2.0 (Bonato et al. 2016). We implemented this also for the already existing literature records, where names were curated to follow currently valid taxonomy. As an exception, *Lithobius jugoslavicus* Matic & Darabantu, 1968 was treated as a synonym of *Lithobius stygius* Latzel, 1880, following the taxonomic status of its substitute name *Lithobius corneliae* Stoev, 1997. Furthermore, *Lithobius punctulatus* was considered a synonym of *Lithobius validus*, following Eason (1972) and Kos (1987). Reassignments of taxa were made in four cases, where reports of species *Eupolybothrus leostygis*, *Eupolybothrus fasciatus*, *Lithobius burzenlandicus wardaranus* and *Lithobius microps* were assigned to *E. obrovensis*, *E. grossipes*, *Lithobius* sp. and *Lithobius carinthiacus*, respectively. The rationale behind these reassignments is explained in the discussion.

Geographic positions of cave entrances were taken from the Cave Registry of Slovenia ("Kataster jam Slovenije"), available at https://www.katasterjam.si/ (Ljubljana Cave Exploration Society 2022), or from GPS coordinates (in cases of caves whose registration is in progress and artificial tunnels). Few localities reported in literature were, due to dubious geographic position, assigned to wider geographic areas or region and not to individual caves. Distributional maps were produced using QGIS, ver. 3.16. (QGIS Development Team 2021).

Data resources

The data underpinning the analysis reported in this paper are deposited at GBIF, the Global Biodiversity Information Facility, and are available at https://www.gbif.org/dataset/2bce5608-b366-4e65-8dbe-6870da7888b2.

Results

Lithobiomorph centipedes were found in 155 subterranean sites (153 caves and two artificial tunnels, Table 1) throughout Slovenia and five less exactly determined subterranean localities (Suppl. material 1). They are reported for the first time from 114 (71%) localities (Fig. 1).

Altogether, 410 occurrence records of lithobiomorph centipedes were gathered, of which 91 (22%) are literature and 319 (78%) new records (Table 1). The records refer to 21 valid species from three genera. Most of the recorded species (18) are known primarily from the surface habitats (Fig. 2), while three of them are considered to be subterranean (Fig. 3).

Table 1. The overview of lithobiomorph centipedes, found in caves of Slovenia. In the column "Literature findings" reports on published records are given, with references and notes on taxa or locality reported in brackets. In the column "New findings", reports on findings that have not been published before are given, with dates, initials of legators and an information whether the animal was found in the part close to the cave entrance (entrance part) or away from the entrance, in the aphotic zone of the cave (deeper part) (when this information was specified in the label). Obligate subterranean species are in cells, shaded in light grey colour. New records come from SubBioDB, with exception of the ones marked with *, that come from ChiloBio dataset. Reported new findings, where the material could not be examined by an expert are marked with #. Coordinates and details on cave positions can be found in the Suppl. material 1. Initials of the legators refer to: AJ – Ana Janović, AK – Anja Kos, Aka – Andrej Kapla, AL – Ana Lozar, AM – Ajda Moškrič, AP – Anja Pekolj, AZ – Aja Zamolo, BR – Behare Rexhepi, BS – Boris Sket, CF – Cene Fišer, DC – David Culver, DK – Darja Kolar, Dku – Džana Kuna, DŠ – David Škufca, EP – Ester Premate, EvP – Eva Pavlovič, FG – Franci Gabrovšek, FK – Franc Kljun, FP – Franc Potočnik, GB – Gregor Bračko, HR – Hans Recknagel, IK – Ivan Kos, JB – Jana Bedek, JJ – Jure Jugovic, JM – Janja Matičič, JS – Janez Stražišar, JZ – J. Zver, KK – Katarina Kanduč, KlK – Klara Kač, LK – Lucija Knauf, LL – Ljerka Lah, LR – Lucija Ramšak, MB – Matej Blatnik, MaP – Matija Perne, MiP – Mitja Prelovšek, MK – Marjeta Konec, MR – Maša Rajh, MS – Marjeta Smrdel, MV – Miloš Vittori, NS – Nataša Sivec, PeP – Petra Pavšič, PG – Primož Gnezda, PK – Peter Kozel, PP – Primož Presetnik, PT – Peter Trontelj, ŠB – Špela Borko, SiP – Simona Prevorčnik, SM – Stefano Mammola, SP – Slavko Polak, TD – Teo Delić, TT – Tjaša Trajbarič, Unk – Unknown, US – Uroš Stepišnik, VS – Valentin Schein, VZ – Valerija Zakšek, ŽF – Žiga Fišer, ŽK – Žan Kuralt, MZ – Maja Zagmajster, UK – Urška Kamenšek.

Taxon name	Literature findings	New findings
Eupolybothrus (*Eupolybothrus*) *grossipes* (C. L. Koch, 1847)	Jama pod južnim vrhom Tisnika (Matic and Stentzer 1977), Jelenska jama (Matic and Dărăbanțu 1968 (as *E. fasciatus*, re-examined in this study)), Razpoka (Matic and Stentzer 1977)	Jama nad Kobilo (10.9.2022, leg. TD), Kadunjška jama (21.6.2014, leg. DŠ, NS), Krivopeta jama (15.5.2022, leg. TD), Mrzla jama pri Ložu (16.7.2007, leg. SP), Predjamski jamski sistem (2.9.2020, leg. PK, deeper part), Rivčja jama (2.8.2021, leg. DŠ, TD, EP)
Eupolybothrus (*Parapolybothrus*) *obrovensis* (Verhoeff, 1930)	Dimnice (Matic and Dărăbanțu 1968; Ozimec and Komerički 2009; Ozimec et al. 2011; Polak et al. 2012 (some references relate to the type locality, given by Verhoeff as "Hohle bei Obrov", Matic and Dărăbanțu (1968) report also on finding from this cave)), Istria (Verhoeff 1933), Medvedjak jama (Attems 1908, 1929 (as *E. leostygis*); Eason 1983; Verhoeff 1933; Wolf 1938 (as *E. obrovensis* and *E. leostygis*); Polak et al. 2012), Pečina v Borštu (Verhoeff 1930; Wolf 1938 (both relate to the type locality, given by Verhoeff as "Hohle bei Obrov")), Polina peč (Polak et al. 2012)	Jama pod Krogom (26.7.2018, leg. EP), Račiška pečina (27.4.2004, leg. SP), Štefakova pečina (24.5.2020, leg. AK, JS, entrance part), Velika Kozinska jama (12.1.2014, leg. MZ, TD)
Eupolybothrus sp.		Jama pod Smoganico (16.9.2020, leg. AK, entrance part), Lisičji grad (25.7.2018, leg. EP), Polina peč (3.2.2006, leg. SP)

Taxon name	Literature findings	New findings
Eupolybothrus (*Leptopolybothrus*) *tridentinus* (Fanzago, 1874)	Jama za Hudim lazom 1 (Kos 1988), Jerinovec (Matic and Dărăbanțu 1968 (as *E. leptopus*)), Mačkovica (Kos 1988), Planinska jama (Matic and Stentzer 1977 (as *E. leptopus*)), Zadlaška jama (Verhoeff 1929 (as *Polybothrus cerberus*, loc. as Dantegrotte bei Tolmin); Manfredi 1932b (as *Polybothrus cerberus*, loc. as Grotta Dante presso Tolmino); Wolf 1938 (as *Polybothrus cerberus*, loc. as Dantegrotte); Eason 1983 (loc. as Grotta Dante near Tolmin)	Babja jama (22.7.2010, leg. MZ), Babja luknja (21.9.2020, leg. AK), Jama nad Kobilo (10.9.2022, leg. TD; 24.2.2007, leg. BS; 27.10.2009, leg. PT, SiP), Jama pod Gradiščarjevim robom (19.6.2009, leg. MZ, JJ), Jama v Lozi pri Orehku (14.5.2020, leg. AK, JS), Kamrica jama (2.7.2021, leg. MZ, TD, EP), Koševka (17.12.2021, leg. EP, BR, entrance part; 13.4.2022, leg. CF, EP, deeper part; 20.4.2022, leg. AK, CF, entrance part; 4.5.2022, leg. BR, AL, TT, entrance part; 27.10.2022, leg. MZ, BR, AK, AP, entrance part), Snežna jama pri Glažuti (22.9.2020, leg. AK, MR), Topli vrh 5 (21.2.2021, leg. TD), Velika Knežja jama (17.5.2020, leg. AK, JS), Željnske jame (22.9.2022, leg. AK, MR), Zelške jame (19.6.2022, leg. AK, entrance part)
Harpolithobius gottscheensis Verhoeff, 1937		Koševka (13.4.2022, leg. CF, EP, entrance part; 10.11.2022, leg. VZ, HR, MZ, entrance part), Topli vrh 5 (21.2.2021, leg. TD), Zavinka jama (26.12.2021, leg. AK, deeper part), Zelške jame (19.6.2022, leg. AK, entrance part)
Lithobiidae	Kubik jama (Polak et al. 2012)	Belojača# (10.11.2003, leg. UK; 18.12.2003, leg. UK, record kept under name *Lithobius* sp.), Kozja luknja# (record kept under name *Lithobius* cf. *stygius*), Jama Tunel v Spodnji Idriji# (27. 10. 2009, leg. MZ, SiP, record kept under name Lithobiomorpha)
Lithobius agilis C.L.Koch, 1847	Babja luknja (Matic and Dărăbanțu 1968), Huda luknja pri Gornjem Doliču (Matic and Stentzer 1977), Jama pod južnim vrhom Tisnika (Novak and Kuštor 1982), Jama pri Votli peči pri Ravnah (Novak and Sivec 1977), Jelenska jama (Matic and Dărăbanțu 1968)), Pilanca (Matic and Stentzer 1977; Novak and Kuštor 1982), Planinska jama (Matic 1979)	Babja luknja (28.3.2006, leg. MiP), Koševka (17.12.2021, leg. EP, BR, entrance part)
Lithobius austriacus (Verhoeff, 1937)		Artificial tunnel at Osek (23.7.2011, leg. MZ)
Lithobius borealis Meinert, 1868		Jama med plastmi (20.7.2020, leg. AK, JS, entrance part)
Lithobius carinthiacus Koren, 1992	Jelenska jama (Matic and Dărăbanțu 1968 (as *L. microps*, re-examined in this study)), Vratnica (Matic and Dărăbanțu 1968 (as *L. burzenlandicus wardaranus*, tentatively assigned, re-examined in this study))	Apolonova jama (17.5.2020, leg. AK, JS, entrance part), Blažev spodmol (26.7.2018, leg. EP), Gašpinova jama (9.7.2021, leg. AK, entrance part), Jama pri jami pri kalih (18.8.2020, leg. AK, JS, entrance part), Koševka (12.7.2022, leg. AK, MZ, Dku, deeper part), Lešanov brezen (11.6.2020, leg. MZ, ŠB, TD, entrance part), Merjaščeva jama (14.6.2020, leg. AK, JS, entrance part), Polharjev kevderc (1.9.2020, leg. AK, JS, entrance part), Skednena jama (18.8.2020, leg. AK, JS)
Lithobius castaneus Newport, 1844		Topli vrh 5 (21.2.2021, leg. TD)
Lithobius dentatus C.L. Koch, 1844		Jamovka (13.10.2022, leg. GB, AP, deeper part)
Lithobius erythrocephalus C.L. Koch, 1847	Carniola (Attems 1959), Hrencova jama (Matic and Dărăbanțu 1968), Istria (Attems 1929 (as *Archilithobius illyricus*)), Jama pod Smoganico (Zapparoli 1989), Pivka jama (Zapparoli 1989), Planinska jama (Zapparoli 1989), Podpeška jama (Zapparoli 1989)	
Lithobius forficatus (Linnaeus, 1758)	Postojnska jama (Wolf 1938), Predjamski jamski system (Wolf 1938 (loc. as Luegger hohlen))	Jama v Pruhu (29.7.2011, leg. MZ)
Lithobius latro Meinert, 1872	Koševka (Matic and Dărăbanțu 1968)	
Lithobius lucifugus L. Koch, 1862	Golobeja jama (Verhoeff 1937a (as *Lithobius microporus*))	

Taxon name	Literature findings	New findings
Lithobius melanops Newport, 1845	Raja peč (Folkmanová 1946 (as *Lithobius glabratus fuscus*, Lok. 776, Vranja pečina))	
Lithobius nodulipes Latzel, 1880		Brezno pri Pojetovih lazih 2 (2.7.2020, leg. AK, JS, entrance part), Doljna vodena jama (20.7.2019, leg. EP), Hajdučka jama (19.11.2022, leg. EP), Jama jugozahodno od Mašuna (29.9.2002, leg. SP), Jama med plastmi (20.7.2020, leg. AK, JS, entrance part), Jama pod Gavgami (3.6.2022, leg. AK, MZ, entrance part), Jama v Mlaki (21.1.2007, leg. SP), Jezerski kevderc (1.9.2020, leg. AK, JS, entrance part), Koševelka (21.6.2020, leg. AK, JS, entrance part; 30.3.2022, leg. AK, MZ, TD, entrance part; 5.4.2022, leg. BR, GB, deeper part; 10.11.2022, leg. VZ, HR, MZ, entrance part), Pajkova Reža (27.10.2014, leg. ŠB), Pečina v Borštu (4.9.2022, leg. AK, entrance part), Pistiškova povšna (20.11.2003, leg. Aka), Polharjev kevderc (1.9.2020, leg. AK, JS, entrance part), Skednena jama (18.8.2020, leg. AK, JS), Slugova jama (26.3.2017, leg. MZ), Špehovka (19.7.2016, leg. EP, AJ), Otoška jama (24.10.2003, leg. LR, entrance part), Trpinova jama (22.5.2022, leg. AK), Tunnel at Spodnja Draga (9.7.2014, leg. DŠ), Velika jama nad Trebnjem (28.7.2009, leg. MZ), Velika Kozinska jama (12.1.2014, leg. MZ, TD), Veliki Hubelj (15.1.2015, leg. ŠB), Vidovec (19.11.2022, leg. EP), Viršnica (22.5.2022, leg. AK), Zadlaška jama (21.7.2010, leg. MZ)
Lithobius sp.	Caves along the underground flow of the river Reka-Timavo (Wolf 1938 (loc. as RekaHohlen)), Mala jama pri Veliki groblji ((Matic and Dărăbanțu 1968 (as *L. burzenlandicus wardaranus,* re-examined in this study)), Račiška pečina (Polak et al. 2012), Tikina jama (Stepišnik and Ramšak 2006)	Brezno na Grmadi (11.3.2007, leg. SP), Čendova jama (3.6.2009, leg. MZ, SiP), Česnovka (14.6.2020, leg. AK, JS), Cikova jama (26.3.2007, leg. SP), Gabrovška jama (17.9.2009, leg. SP, VS; 8.9.2009, leg. SP), Jakobova luknja (2.9.2020, leg. AK), Jama jugozahodno od Mašuna (29.9.2002, leg. SP), Jama na Opalah (24.7.2020, leg. PP, EP), Jama na Pucovem Kuclu (25.7.2020, leg. MaP, PP, EP), Jama pod Gavgami (3.6.2022, leg. AK, MZ, entrance part), Jama pod Krogom (6.6.2021, leg. AK, entrance part), Jama pod Smoganico (16.9.2020, leg. AK, entrance part), Jama v doktorjevi ogradi (25.11.2021, leg. MZ, CF, ŠB, EP), Jama v Sodolih (2.7.2020, leg. AK, JS), Jerinovec (21.6.2020, leg. AK, JS, entrance part; 20.4.2022, leg. BR, AL; 4.5.2022, leg. AK, EP, BR, TT, AL, deeper part; 6.10.2022, leg. AK, BR, deeper part), Kamrica jama (5.1.2021, leg. TD), Koševelka (21.6.2020, leg. AK, JS, entrance part; 10.12.2021, leg. TD, CF, deeper part; 7.1.2022, leg. Unk, entrance part; 30.3.2022, leg. AK, MZ, TD, entrance and deeper part; 5.4.2022, leg. BR, GB, entrance and deeper part; 20.4.2022, leg. AK, CF, deeper part; 4.5.2022, BR, TT, AL, entrance part; 19.7.2022, leg. AK, GB, entrance part; 26.7.2022, leg. TD, AP, deeper part; 9.8.2022, leg. EP, CF, entrance part; 13.10.2022, leg. MZ, ŠB, SM, AP, GB, deeper part; 20.10.2022, leg. MZ, BR, EP, entrance part; 10.11.2022, leg. VZ, HR, MZ, entrance and deeper part), Mačkovica (20.10.2022, leg. AK, entrance part; May 1970, leg. Unk*; 27.12.1969, leg. Unk*; 25.7.1969, leg. Unk*), Mali Obrh spring (12.10.2022, leg. SP), Mivči kevderc (16.9.2009, leg. SP, VS), Mrzla jama pri Ložu (16.7.2007, leg. SP), Otoška jama (12.9.2003, leg. LR, deeper part; 3.10.2003, leg. LR, entrance part; 29.12.2003, leg. LR, deeper part; 16.1.2004, leg. LR, entrance part; 19.3.2004, leg. LR, deeper part; 23.7.2004, leg. LR, entrance part; 13.8.2004, leg. LR, deeper part; 3.9.2004, leg. LR, entrance and deeper part; 24.9.2004 leg. LR, deeper part), Pajkova Reža (27.10.2014, leg. ŠB), Paščipajkovka (2.7.2022, leg. AK, entrance part), Pečina v Borštu (4.9.2022, leg. AK), Pistiškova povšna (20.11.2003, leg. Aka), Planinska jama (4.4.2006, leg. SP; 20.10.2010, leg. MV, TD, MK, JJ), Postojnska jama (12.9.2003, leg. LR, deeper part; 14.11.2003, leg. LR, entrance part; 29.12.2003, leg. LR, deeper part; 6.2.2004, leg. LR, entrance and deeper part; 21.5.2004, leg. LR, entrance and deeper part; 23.7.2004, leg. LR, entrance part; 13.8.2004, leg. LR, entrance part; 3.9.2004, leg. LR, entrance part; 24.9.2004 leg. LR, entrance and deeper part; 14.12.2016, leg. PK, MB, entrance part), Praprotno (3.5.2007, leg. SP), Predjamski jamski sistem (2.9.2020, leg. PK, entrance part; 2.12.2020, leg. PK, entrance part; 19.5.2022, leg. PK, KK, deeper part), Prva nižinska (25.11.2020, leg. TD, ŠB), Raja peč (29.7.2009, leg. MZ), Risova jama (26.6.2022, leg. AK, JS), Slugova jama (11.4.2007, leg. SP), Strmška jama (20.10.2009, leg. SP), Svinjska jama pri Dolenji vasi (23.1.2017, leg. MZ, FK), Škocjanske jame (26.9.2018, PK, entrance part), Tkavčja jama (1.9.2022, leg. AK), Tomažinov brezen (14.6.2020, leg. AK, JS), Trpinova jama (22.5.2022, leg. AK), Vančeva jama (5.11.2004, leg. MZ, MS, LR), Vodovodna jama (15.9.2020, leg. AK, entrance part; 26.7.2010, leg. MZ), Zadlaška jama (21.7.2010, leg. MZ)

Taxon name	Literature findings	New findings
Lithobius stygius Latzel, 1880	Carniola (Attems 1959), Jama 1 v Mahovniku (Matic and Dărăbanţu 1968), Jama pod Smoganico (Verhoeff 1929; Manfredi 1932b; Wolf 1938), Lukova jama pri Zdihovem (Matic and Dărăbanţu 1968), Mačkovica (Verhoeff 1929; Wolf 1938 (as Mackovo jama)), Magdalena jama (Wolf 1938), Mrzla jama pri Ložu (Polak and Pipan 2021), Planinska jama (Latzel 1880; Attems 1929; Matic and Dărăbanţu 1968; Matic and Stentzer 1977; Matic 1979; Zapparoli 1989), Podpeška jama (Matic and Dărăbanţu 1968 (as *L. stygius* and *L. jugoslavicus*)), Postojnska jama (Latzel 1880; Attems 1929; Manfredi 1932b; Wolf 1938; Matic 1978), Predjamski jamski sistem (Wolf 1938 (loc. as FurstWindischgratzerhohle)), Tekavčja jama (Matic and Dărăbanţu 1968), Velika Karlovica (Matic and Dărăbanţu 1968), Vodna jama (Matic and Dărăbanţu 1968), Zadlaška jama (Verhoeff 1929; Manfredi 1932b; Wolf 1938 (all references loc. as Dantegrotte)), Željnske jame (Matic and Dărăbanţu 1968)	Apolonova jama (17.5.2020, leg. AK, JS, entrance part), Brezno pod Jelen brdom (28.6.2003, leg. FK*), Brezno pri Pojetovih lazih 2 (2.7.2020, leg. AK, JS, entrance part), Črna jama (16.8.2020, leg. PG), Dolga jama pri Koblarjih (20.4.2012, leg. TD; 23.11.2004, leg. MZ, LR, US), Doljna vodena jama (19.5.2020, leg. AK, JS), Drežniška jama (28.2.2016, leg. TD), Fosilni rov 1 (25.7.2021, leg. AK, entrance part), Fučkovski zdenec (23.7.2015, leg. ŽF), Gornja vodena jama (19.5.2020, leg. AK, JS), Jama 1 pri Planinski jami (29.10.2022, leg. MZ, AK, entrance and deeper part), Jama 1 v Mahovniku (24.1.20211, leg. AK, JS), Jama na vzhodnem pobočju Roga (16.5.2020, leg. AK), Jama pod cesto (22.12.2010, leg. Unk), Jama Sv. Loja (17.5.2011, leg. TD), Jama Treh bratov (27.6.2021, leg. AK, entrance part), Jama v kamnolomu (6.9.2005, leg. SP; 27.7.2015, leg. ŽF; 24.8.2006, leg. PT, VZ), Jama v Suhi Rebri (19.4.2020, leg. MZ, ŠB, TD), Jama v Taborski steni (24.9.2003, leg. SP), Jama zahodno od Škrilj (5.4.2020, leg. IK, AK, entrance part), Jelovička jama (21.7.2014, leg. TD), Kobilna jama (2.12.2017, leg. EP), Lobašgrote (24.7.2014, leg. TD; 20.9.2004, leg. PT; 22.9.2020, leg. AK, MR, entrance and deeper part), Lukova jama pri Zdihovem (20.11.2003, leg. BS, SP, DC), Mala Skednenca (26.7.1994, leg. SP*), Mali džot (5.4.2007, leg. SP), Mali Obrh spring (12.10.2022, leg. SP), Mrzla jama pri Ložu (13.10.2022, leg. HR, SM; 16.7.2007, leg. SP), Otoška jama (12.9.2003, leg. LR, deeper part; 24.10.2003, leg. LR, deeper part; 8.12.2003, leg. LR, deeper part; 29.12.2003, leg. LR, deeper part; 16.1.2004, leg. LR, entrance part; 6.2.2004, leg. LR, deeper part; 27.2.2004, leg. LR, deeper part; 19.3.2004, leg. LR, deeper part; 30.4.2004, leg. LR, deeper part; 21.5.2004, leg. LR, entrance and deeper part; 11.6.2004, leg. LR, deeper part; 13.8.2004, leg. LR, deeper part; 3.9.2004, leg. LR, deeper part), Paščipajkovka (2.7.2022, leg. AK, entrance part), Pikčeva jama (13.4.2020, leg. IK, AK; 31.10.1993, leg. IK*), Planinska jama (29.10.2022, leg. MZ, JB, AK, LK, deeper part; 24.11.2009, leg. MK, JJ, AM; 27.4.2020, leg. MZ; 24.4.1986, leg. FP*), Podpeška jama (15.1.2018, leg. IK, ŽK; 24.1.1989, leg. FP*), Postojnska jama (12.9.2003, leg. LR, entrance and deeper part; 3.10.2003, leg. LR, entrance and deeper part; 24.10.2003, leg. LR, entrance and deeper part; 14.11.2003, leg. LR, entrance part; 8.12.2003, leg. LR, entrance and deeper part; 16.1.2004, leg. LR, entrance and deeper part; 27.2.2004, leg. LR, deeper part; 19.3.2004, leg. LR, deeper part; 9.4.2004, leg. LR, entrance part; 30.4.2004, leg. LR, entrance and deeper part; 21.5.2004, leg. LR; 11.6.2004, leg. LR, entrance part; 2.7.2004, leg. LR, entrance and deeper part; 23.7.2004, leg. LR, deeper part; 13.8.2004, leg. LR, entrance part; 3.9.2004, leg. LR, entrance and deeper part; 24.9.2004, leg. LR, entrance part; 14.12.2016, leg. PK, MB, entrance part; 10.2.2022, leg. PK, KK, entrance part; 29.9.2022, leg. PK, PG, entrance part; 20.10.2022, leg. AK, entrance part), Prvoaprilska jama 2 (22.9.2020, leg. AK, MR), Rauhova jama (26.2.2016, leg. TD, EP, DŠ, DK, PeP, NS), Risova jama (26.6.2022, leg. AK, JS), Šepčev skedenj (19.5.2020, leg. AK, JS), Skednevnica (22.5.2022, leg. AK, deeper part), Škratovka (30.9.2016, leg. PT), Snežna jama pri Glažuti (22.9.2020, leg. AK, MR), Strmška jama (20.10.2009, leg. SP), Svinjska jama pri Dolenji vasi (5.1.2011, leg. MZ, KIK, JM, TD), Trpinova jama (22.5.2022, leg. AK), Vančeva jama (23.10.2010, leg. MZ, SiP, TD; 9.5.2010, leg. MZ; 23.11.2004, leg. MZ, US, LR), Velika Karlovica (28.7.1994, leg. SP*), Velika Knežja jama (17.5.2020, leg. AK, JS), Velika Skednenca (28.7.1994, leg. SP*), Veliki kevder v Bukovju (22.5.2022, leg. AK, entrance part), Vetrovna jama pri Laški kukavi (5.12.2013, leg. MZ, PT, TD), Željnske jame (22.9.2022, leg. AK, MR), Zelške jame (8.8.2003, leg. MZ, PT, LL), Zijavka (3.5.2007, leg. SP)
Lithobius tenebrosus Meinert, 1872		Košelevka (13.4.2022, leg. CF, EP, entrance part), Zapostavljena jama (31.7.2020, leg. AK, JS, entrance part)
Lithobius tricuspis Meinert, 1872		Brezno pri Pojetovih lazih 2 (2.7.2020, leg. AK, JS, entrance part), Čendova jama (16.9.2020, leg. AK, entrance part), Ciganska jama pri Predgrižah (22.6.2011, leg. TD, KIK, JM, JJ), Jama med plastmi (20.7.2020, leg. AK, JS, entrance part), Jama na Pucovem Kuclu (25.7.2020, leg. MaP, PP, EP), Jama nad izvirom pri Šinkovčevi žagi (12.5.2009, leg. MZ, SiP), Jama pri Glažuti (22.9.2020, leg. AK, MR), Jama v gradu pri Osojnici (21.7.2020, leg. EvP, PP, EP, TT), Jelenska zijalka (5.8.2022, leg. TD, AZ), Jerinovec (21.6.2020, leg. AK, JS, entrance part), Košelevka (21.6.2020, leg. AK, JS, entrance part; 13.4.2022, leg. CF, EP, entrance part; 12.7.2022, leg. AP, MZ, Dku, entrance part; 19.7.2022, leg. AK, GB, entrance part), Pilanca (19.7.2016, leg. ŽF), Polharjev kevderc (1.9.2020, leg. AK, JS), Predjamski jamski sistem (18.6.2020, leg. PK, deeper part; 2.9.2020, leg. AK; 2.9.2020, leg. PK, entrance and deeper part; 2.12.2020, leg. PK, entrance and deeper part; 27.1.2021, leg. PK, deeper part; 18.11.2021, leg. PK, PG, deeper part; 10.2.2022, leg. PK, KK, entrance and deeper part; 19.5.2022, leg. PK, KK, deeper part; 7.7.2022, leg. PK, deeper part; 29.9.2022, leg. PK, PG, deeper part), Raja peč (29.7.2009, leg. MZ), Ravensko brezno (22.7.2009, leg. MZ, PT), Ruščeva jama (1.6.2009, leg. MZ, PT), Spodmol v Brezovcu (22.8.2003, leg. SP), Zelške jame (19.6.2022, leg. AK, entrance part)

Taxon name	Literature findings	New findings
Lithobius validus Meinert, 1872	Caves along the underground flow of the river Reka-Timavo (Wolf 1938 (loc. as RekaHohlen)), Huda luknja pri Radljah (Matic and Stentzer 1977 (as *Lithobius p. punctulatus*)), Kačna jama (Kos 1987), Mačkovica (Matic 1979 (as *L. punctulatus*)), Škocjanske jame (Matic and Dărăbanţu 1968; Polak 2017)	Jama 1 v Kanjaducah (5.9.2013, leg. TD, FG; 13.5.2015, leg. TD; 11.12.2005, leg. PT), Kačna jama (25.9.2013, leg. TD, FG), Košelevka (21.6.2020, leg. AK, JS, entrance part; 13.4.2022, leg. CF, EP, entrance part), Okno jama (19.7.2021, leg. EP), Predjamski jamski sistem (3.12.2019, leg. PK, deeper part; 18.6.2020, leg. PK, deeper part; 2.9.2020, leg. AK; 2.9.2020, leg. PK, entrance and deeper part; 2.12.2020, leg. PK, entrance and deeper part; 27.1.2021, leg. PK, entrance part; 8.7.2021, leg. PK, PG, entrance part; 18.11.2021, leg. PK, PG, entrance and deeper part; 7.7.2022, leg. PK, entrance and deeper part; 29.9.2022, leg. PK, PG, entrance and deeper part), Škocjanske jame (4.10.2017, leg. PK, entrance part; 6.12.2017, leg. PK, entrance part), Tomažinov brezen (14.6.2020, leg. AK, JS, entrance part), Trpinova jama (22.5.2022, leg. AK)
Lithobius zveri (Matic & Stenzer, 1977)	Planinska jama (Matic and Stentzer 1977), Slovenia, in caves (Stojanović et al. 2021)	

Figure 1. Localities with records of lithobiomorph species in Slovenia. The black dots represent localities, from which lithobiomorph centipedes are reported for the first time, while the white circles represent existing literature records.

Compiled data show that *Lithobius stygius* is the most commonly found species and was reported in 60 sites, presenting almost a third of all reported findings. Species *L. tricuspis*, *L. nodulipes* and *Eupolybothrus tridentinus* were also very common (Figs 2, 3), reported in more than 15 locations. On the other hand, eight species are reported only from a single locality, namely *Lithobius castaneus*, *L. austriacus*, *L. borealis*, *L. latro*, *L. lucifugus*, *L. melanops*, *L. dentatus* and *L. zveri* (Table 1).
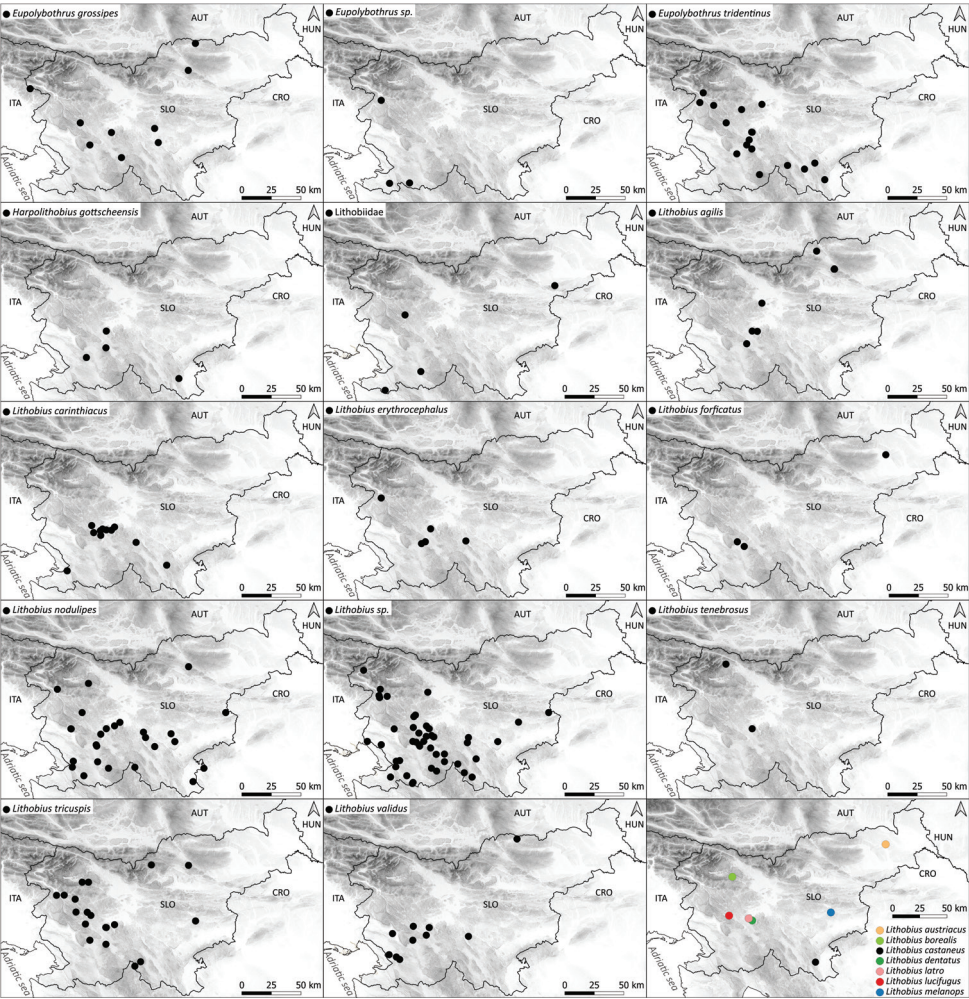
Figure 2. Recorded localities of the surface lithobiomorph centipede species from Slovenian caves.

Discussion

Three lithobiomorph centipede genera, comprising at least 21 species can be found in caves of Slovenia. In addition, almost half of the lithobiomorph species found in Slovenia (Kos 1992a; Stoev 1997) were found in caves at least once. Systematic collecting contributed to a significant increase in the knowledge on centipedes from caves, as 78% the of presented findings are new. Nine species, all of them considered surface and occurring in caves only accidentally, are reported for the first time from Slovenian caves; *Harpolithobius gottscheensis*, *Lithobius austriacus*, *L. borealis*, *L. castaneus*, *L. carinthiacus*, *L. dentatus*, *L. nodulipes*, *L. tenebrosus* and *L. tricuspis*.
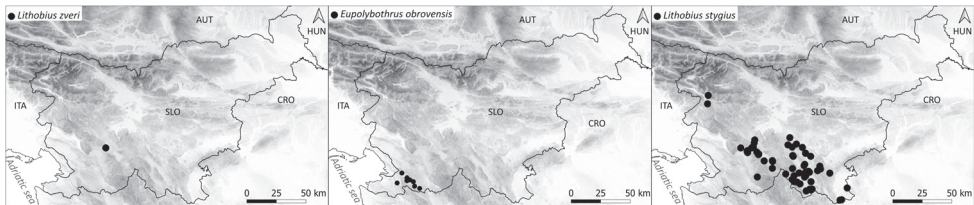
Figure 3. Recorded localities of the troglobiont lithobiomorph centipede species from Slovenian caves.

Records of many centipede species from caves opens a series of questions on the differences of their habitat preferences towards subterranean habitats. Most of the recorded specimens from our dataset belong to surface species, but it is unclear whether these species occur in caves only accidentally, occasionally, or caves present a regular part of their habitat. Some species were found only in a few caves, indicating their accidental occurrence there, similar to what was found in proturans (Galli et al. 2021). On the other hand, some species were frequently found in caves, which may indicate their active use. Different preferences towards caves between species are partly supported also by other studies, e.g., genus *Eupolybothrus* is known to have many species occurring often or solely in caves (Stoev et al. 2010; Akkari et al. 2017). The species *E. obrovensis*, *L. stygius* and *L. zveri* have so far not been found outside caves and are thus considered troglobiotic (Sket 2008; Ozimec and Komerički 2009; Zagmajster et al. 2021). On the other hand, strict delimitation between caves and surface habitats is not unambiguous. The turnover is gradual and continuous, and includes many cracks and fissures in the epikarst (Culver and Pipan 2008, 2014) – a transition zone between surface and deeper subterranean habitats (Prous et al. 2004; Kozel et al. 2019). Finally, the caves present only a sampling point that is directly accessible to humans. In several cases, the specimens were found in the entrance parts of caves, where environmental conditions are similar in many aspects to those in the upper soil layers (e.g., high availability of organic matter and high humidity) which are primary habitat for centipedes and other edaphic organisms (Voigtländer 2011). The connectivity of those habitats is indicated also by our results, as many surface centipedes were recorded despite the assumption that caves are not their main habitat. Therefore, their findings likely also reflect the ecological conditions around the caves and their geographic position. One interesting example is the finding of the species *L. austriacus* in an artificial tunnel in Osek in Northeast Slovenia. This species is known only from mostly non-karstic Northeastern part of Slovenia and countries to the East and North (Simaiakis and Strona 2015). On the other hand, species *L. borealis* is generally found in higher altitudes, as was also the case in our finding.. Interestingly, species *E. tridentinus*, *L. nodulipes*, *L. stygius and L. tricuspis* were found in caves which are located in an altitudinal span of approximately 1000 m a.s.l. This might not be surprising as those were the most commonly recorded species.

Taxonomic comments

The compilation of occurrence dataset inevitably relates to the current knowledge on lithobiomorph centipede taxonomy, which is incomplete, prone to temporal changes, and additionally complicated by varying taxonomic interpretations between the researchers as several taxa need revision (Zapparoli 2003; Bonato et al. 2011). Due to mentioned shortcomings the comparison of literature records is challenging and, the taxonomical curation of the dataset was necessary to include currently valid names and to address possible misidentifications originating from different taxonomic concept. As example, some specimens cited in Matic and Dărăbanţu, (1968) where re-examination of material was possible, were assigned to different species; reported finding of *E. fasciatus* and *L. microps*, correspond to *E. grossipes* and *L. carinthiacus*, respectively. Determinations of specimens reported as *L. burzenlandicus wardaranus* could not be conclusive due to damaged material, but the finding from Mala jama pri Veliki Groblji corresponds to a yet undescribed taxon *Lithobius anici* nomen nudum, reported from several surface sites in Slovenia (Kos 1995; Kuralt et al. 2022). Similarly, the finding from Vratnica likely belongs to *Lithobius carinthiacus* (due to presence of three spines on ventral side of 15th prefemur), but specimen is too damaged to make conclusive determination. Furthermore, findings of *Eupolybothrus leostygis*, reported by some authors, were corrected to *E. obrovensis* as suggested by Verhoeff (1934) and Eason (1983). Interpretation of taxonomic status for *Lithobius microporus* Verhoeff, 1937, *Lithobius jugoslavicus* Matic & Dărăbanţu, 1968 and *Lithobius glabratus fuscus* Folkmanova, 1946 is treated as dubious. They are considered as probable synonyms of *Lithobius lucifugus* L. Koch, 1862, *Lithobius stygius* Latzel, 1880 and *Lithobius melanops* Newport, 1845 respectively (Stoev 2001; Bonato et al. 2016); however, their identity was not thoroughly studied. Similarly, synonymy of *L. illyricus* Latzel, 1880 with *L. erythrocephalus* C.L. Koch, 1847 is stated in ChiloBase 2.0, however it was never formally established and the species is considered valid by some authors (Kos 1992a; Stoev 1997; Stagl and Zapparoli 2006). On the other hand, Zapparoli (1989), who reported *L. erythrocephalus* from caves in Slovenia, considered that characters of *L. illyricus* are within the variation of *L. erythrocephalus*. Existence of potential new species, some of which might represent morphologically identical, cryptic species, additionally hinders complete overview of the species diversity. Their potential presence was detected among the examined individuals in our study and was also reported in literature (Zagmajster et al. 2021). This is expected as there have been descriptions of new species from the Dinaric caves in recent time (Stoev et al. 2015; Akkari et al. 2017; Stojanović et al. 2021) and cryptic diversity is a common problem in many centipede groups (Spelda et al. 2011; Del Latte et al. 2015; Kuralt et al. 2022; Peretti et al. 2022).

Taxonomic knowledge of several lithobiomorph species that were described from Slovenian caves changed over time. Eyeless centipede, *Eupolybothrus obrovensis* (Fig. 4) was described based on subadult male under the name *Lithobius obrovensis*. Species type locality is "Höhle von Obrovo" (Verhoeff 1930), interpreted as Dimnice cave by most of the subsequent authors (Matic and Dărăbanţu 1968; Ozimec and Komerički

Figure 4. Three lithobiomorph centipede species found in Slovenian caves. Subterranean centipede *Lithobius stygius* (upper left), surface species *Lithobius nodulipes* (upper right) and subterranean species *Eupolybothrus obrovensis* (bottom) (Photo: Teo Delić).

2009) or as Pečina v Borštu (Wolf 1938; this article). Later work by Verhoeff (1933) provides information on an adult male from Medvedjak and Matic & Daranbatu (1968) provided a description of a female from Dimnice. Nevertheless, a redescription of the species, based on the adult male and the consolidation of the type locality is needed. Attems (1908, 1929) found the species even before it was formally described in 1930, proclaiming it for *Eupolybothrus leostygis*, species which is otherwise narrowly distributed in Southern Croatia and Bosnia and Herzegovina. This error is mentioned already by Verhoeff (1934), however, several other authors (Manfredi 1932b; Wolf 1938) cited this finding, and species *E. leostygis* ended up even in the checklist of centipedes in Slovenia (Kos 1992a). In addition, the locality name Medvedova jama (referring to cave Medvedjak, Cad. nr. 881, "bear cave") was translated into Italian "Grotta dell'Orso" and misinterpreted as a cave near Gabrovizza, San Primo in Italy (Manfredi 1932a, 1932b; Wolf 1938; Eason 1983). *Eupolybothrus obrovensis* was long known only from few caves, but the speleobiological surveys after the year 2000 yielded more findings, improving our knowledge on the species's narrow distribution. The species occurs in Primorska and Istria regions in Slovenia and Croatia (Table 1; Ozimec and Komerički 2009). The species is listed as Vulnerable on the Slovenian and Croatian Red List (Kos 1992b; Wraber et al. 2002; Ozimec and Komerički 2009) and is protected species by the Croatian national legislation (Official Gazette of the Repub-

lic of Croatia No. 70/05, 139/08). Presumably, the species is vulnerable to changes in habitat and cave fauna, especially due to its narrow distribution and high position in the food chain (Ozimec and Komerički 2009).

Species with the highest number of occurrences, *Lithobius stygius* (Fig. 4), was described from Postojnska jama by Latzel (1880) and reported in caves through the Balkans (Stoev 2001). The existing reports are subjected to some taxonomical unclarities, emphasising the need for its further study. Examples from the area of Slovenia include reported species *L. erythrocephalus* and *L. illyricus*, surface species similar to *L. stygius*, from several caves (Attems 1929; Matic and Dărăbanţu 1968; Zapparoli 1989; Gasparo 1995, 1998). It is unclear whether all of them are truly correct identifications or possible misidentifications, and refer to findings of *L. stygius* or another, similar, and potentially new species. On the other hand, species *Lithobius jugoslavicus* Matic & Dărăbanţu, 1968 is one of the possible junior synonyms of *L. stygius*, where revision is needed to clarify its status (Stoev 2001). It is very likely that the species was described based on an abnormal individual. In some of the works it was referred also as *Lithobius corneliae* Stoev, 1997 in attempt to distinguish it from *Lithobius jugoslavicus* (Hoffer, 1937) (now a synonym of *Lithobius matulici* Verhoeff, 1899, occurring in Bosnia and Herzegovina) (Stoev 1997; Dányi et al. 2019). Preliminary genetic analyses covering the North-western Dinarides showed that the species *L. stygius* is occurring in the south-eastern part of Slovenia and north-western Croatia, while there exists also morphologically similar, but genetically distinct species (Kos A., unpublished). Furthermore, there have been records of synonyms *Lithobius cerberi* Verhoeff, 1943 and *Oligobothrus luciani* Folkmanová, 1935 in the Balkans (Folkmanova 1935; Verhoeff 1943; Eason 1983; Stoev 2001), as well as possible synonym *L. temnensis* Verhoeff, 1943, which was reported from caves as well as surface (Zapparoli 2002).

Lithobius zveri is eyeless centipede and was described by Matic and Stenzer (1977). Even though it was found in one of the most surveyed caves, Planinska jama, it is known only from a single specimen. This might be due to its rarity, our inability to sample its main habitat, small size or lack of targeted sampling. The species is morphologically well separated from other species (Stojanović et al. 2021), however further findings on this elusive and presumably highly endemic species are needed to better understand its taxonomic position.

Polybothrus cerberus Verhoeff, 1929, *Lithobius microporus* Verhoeff, 1937 and *Lithobius glabratus fuscus* Folkmanova, 1946 were also described from Slovenian caves, but are now considered as synonyms of *Eupolybothrus tridentinus* (Fanzago, 1874), *Lithobius lucifugus* L. Koch, 1862 and *Lithobius melanops* Newport, 1845, respectively (Eason 1983; Zapparoli 1989; Stoev 1997). To make it even more complicated the identity of the type locality for the subspecies *Lithobius glabratus fuscus*, described as Lok. 776 Vranja peć, is unclear. Stoev (1997) suggested that the locality might be in Croatia. Naming of the localities follows the collection Biospeleologica balcanica, enabling a direct comparison with other literature. Willmann (1941) describes this locality as Vranja peć bei Saxenstein, Carniola. Based on an examination of old names for toponyms and settlements in Slovenia and Croatia, we concluded that this cave is most likely Raja peč (Cad. nr. 371, one of its synonyms is Vranja pečina) near town

Boštanj, which was referred to as Savenstein in German (the name was apparently misspelt by researchers).

Open challenges

Occurrence and distribution of lithobiomorph centipedes in Slovenia open many interesting questions and directions for further studies. The frequent findings of different surface centipede species underground open question on the connectivity of subterranean and surface habitats. It remains open to investigate, how the species composition in caves is related to the overall centipede species diversity in the region. Further on, lithobiomorph centipedes may present an interesting group to study processes related to colonization of subterranean habitats, especially as there are groups of species that can be found in the surface-subterranean ecotone as well as deep in caves.

As top predators and K-strategists (Albert 1983; Vahtera et al. 2020), lithobiomorph centipedes might have an specific position in subterranean ecosystems, however, their role in communities is still understudied. Different surface species might be part of different communities and are presumably more interconnected with community at cave entrances, while exclusively subterranean species are likely part of the communities deeper in caves (Novak et al. 2012; Cuff et al. 2021). In the Dinarides, there are several centipede species that were found in deep parts of caves, with geophilid centipede *Geophilus hadesi* Stoev, Akkari, Komerički, Edgecombe & Bonato, 2015 holding depth record with finding in cave Lukina jama – Trojama cave system, at depth 1100 m (Stoev et al. 2015). Given the lack of ecological data, further studies would be needed to investigate the ecology of centipedes and their role in subterranean communities.

This paper represents an important contribution to the knowledge of diversity of centipedes in the caves of Slovenia. It is clear that centipedes are a diverse group of animals that, despite the rich history of research require further faunistic and ecological studies to gain at least a basic understanding of their role in communities within subterranean realm. With the data at hand, the understanding of their taxonomy is also far from complete, as is evident from the existence of numerous taxonomic issues. Therefore, further work should aim to resolve taxonomic status, include molecular information on the specimens to improve the resolution of centipede species diversity and their phylogenetic relationships, and to set a solid framework for future ecological studies.

Acknowledgements

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Supplementary material 1

Detailed information on localities with reported lithobiomorph centipedes

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Data type: table (.xlsx file)

Explanation note: Locality name is provided, followed by the National Cave Registry number, closest and largest settlement, latitude and longitude, altitude (rounded to 10 m), number of recorded taxa and number of taxa with first report on the occurrence from the cave (number of taxa was determined as minimal number, counting only lowest determined categories in case of e.g. both species and genus level determinations), information whether this work is reporting on lithobiomorph centipedes from the locality for the first time and additional comments (e.g. synonyms reported in the cited resources).

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SHORT COMMUNICATION

First record of a freshwater cave sponge (Porifera, unknown gen. and sp.) in a cave inhabited by *Astyanax* cavefish in the Sierra de El Abra, San Luis Potosí, Mexico

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Abstract

The karstic cave, la Cueva de Los Sabinos, located in the Sierra de El Abra in the state of San Luis Potosí, Mexico, is mostly known for hosting a population of blind, depigmented *Astyanax mexicanus* cavefish. Herein, we report the discovery of a non-pigmented sponge (Porifera) in the final sump of this cave. No genus or species name could be attributed because we did not collect any specimen. Up to now, the sponge distribution seems restricted to a single pool in la Cueva de Los Sabinos, but further careful exploration of other pools of the cave as well as closely related cavities is warranted. To our knowledge, this observation constitutes the fourth report of a freshwater, white, cave-adapted sponge in the world and the first for Mexico and North America. It is also the eleventh troglobite species encountered in Los Sabinos. Our discovery confirms the exceptionally rich biodiversity of this cave ecosystem.

Resumen

La cueva kárstica de Los Sabinos, localizada en la Sierra de El Abra en el Estado de San Luis Potosí, México, es principalmente conocida por albergar una población de peces de cueva *Astyanax mexicanus*, ciegos y despigmentados. Aquí, reportamos el descubrimiento de una esponja despigmentada (Porifera) en el sifón final de esta cueva. No se pudo asignar ningún nombre de género o especie porque no se colectó ningún espécimen. Hasta ahora, la distribución de la esponja parece restringida a un único estanque de la cueva de Los Sabinos, pero será necesaria una exploración más detallada y cuidadosa de otros cuerpos de agua de la cueva, así como de las cavidades aledañas con posible conexión con esta cueva. Hasta donde sabemos, esta observación constituye el cuarto reporte de una esponja de agua dulce, no pigmentada y adaptada a cuevas en el mundo y la primera para México y Norteamérica. También es la undécima especie troglobia que se ha encontrado en la cueva de Los Sabinos. Nuestro descubrimiento confirma la excepcional riqueza en biodiversidad de este ecosistema cavernícola.

Introduction

Sponges (Porifera) are very common organisms in marine environment with more than 9000 valid species from around the world (de Voogd et al. 2023). These metazoans are less known and studied in freshwater systems, yet they appear relatively common in different continental water habitats like rivers and lakes (Manconi and Pronzato 2008; Evans 2016), except in Antarctica. In the Neotropical region, Manconi and Pronzato (2008, 2015) reported 73 species in 26 genera of Spongillina (Class Demospongiae) in freshwater, among a total of 238 species around the world. Today, the number of species probably approximates 250, as new species are regularly discovered (Nicacio et al. 2011; Hernández and Barreat 2017; Carballo et al. 2018; Gómez 2011; Briceño et al. 2020; Carballo et al. 2021). New occurrences of previously described species are often reported (Rueda and Mesquita-Joanes 2016; Briceño et al. 2020). The taxonomic classification is rapidly evolving and complex (Sollas 1885; Manconi and Pronzato 2002; Cândido et al. 2010; Annesley and Henderson 2011).

In Mexico, nine species of freshwater sponges are listed in the World Porifera Database (https://www.marinespecies.org/porifera/distribution.php?p=details&id=2224) and in WoRMS (World Register of Marine Species). For most of them, occurrences are old in literature and need revision (Potts 1885, 1885b; Martinez 1940; Rioja 1940a, 1942; Penney and Racek 1968; Bushnell 1971; Poirrier 1982). In addition, four other species (not listed on WPDatabase for presence in Mexico) could be present according to old literature as well (Ehrenberg 1841; Old 1936; Rioja 1940b, 1940c, 1953a, 1953b; Poirrier 1982). Moreover, three species have been described very recently (Carballo et al. 2018, 2021; Gómez et al. 2019). A complete discussion of the freshwater sponge species encountered in Mexico can be found in Gómez-López (2011).

There are, on the other hand, very few records of cave-adapted, strictly freshwater sponges throughout the world. Of note, marine or anchialine sponges can be found in sub-

terranean habitats, but most of them are not specific, cave-adapted animals. Strictly freshwater, troglobite, non-pigmented sponges are rarely found, maybe in part due to the difficulties in exploring and surveying these extreme underground habitats. Two cave sponges were described so far in Brazil: *Arinosaster patriciae* Volkmer-Ribeiro, Tavares-Frigo, Ribeiro & Bichuette, 2021 from the Arinos River basin, state of Mato Grosso and *Racekiela cavernicola* Volkmer-Ribeiro, Bichuette & de Sousa Machado, 2010 in a large cave system in the state of Bahia (Volkmer-Ribeiro et al. 2010, 2021). In Europe, the species *Eunapius subterraneus* (Sket & Velikonja, 1984) that includes two subspecies, *E. s. subterraneus* and *E. s. mollisparspanis*, was reported in Croatia (Gottstein Matočec et al. 2002; Bedek et al 2008; Harcet et al. 2010; Jalžić et al. 2013). To our knowledge, these three species constitute the only records of sponges in karstic freshwater caves (Trajano and Bichuette 2010; Volkmer-Ribeiro et al. 2021), and no species has been described in Mexico (Reddell 1981).

Here, we document the discovery of a subterranean freshwater sponge in a cave inhabited by troglomorphic *Astyanax mexicanus* (De Filippi, 1853) in the state of San Luis Potosi, Mexico (Fig. 1A). The region is famous for the presence of at least 33 caves hosting troglomorphic populations of *Astyanax mexicanus/jordani* complex (Elliott 2018; Espinasa et al. 2018, 2020; Miranda-Gamoa et al. 2023). La Cueva de Los Sabinos, located in the Sierra de El Abra was first discovered in 1942 (Mitchell et al. 1977). It is a well-known cave that has been studied extensively by cavers and biologists (Elliott 2018). Sixty animal species belonging to diverse phyla have been inventoried, including nine troglobite species. However, no sponge has been recorded yet (Elliott 2018). According to Elliott (2018), the nine troglobite species in Los Sabinos are:

1. *Microdiaptomus cokeri* (Osorio-Tafall, 1942): Crustacea, Copepoda, Diaptomidae.
2. *Hobbsiella cirolanae* (Rioja, 1951): commensal ostracod crustacean, known from 15 caves in the Sierra de El Abra and Sierra de Guatemala.
3. *Speocirolana pelaezi* Bolivar & Pieltai, 1950: aquatic isopod crustacean.
4. *Pseudosinella strinatii* (Gisin, 1952): collembolan (springtail) known from nine caves in the Sierra de El Abra.
5. *Spherarmadillo cavernicola* Mulaik, 1960: pillbug (terrestrial isopod crustacean) from the El Abra and Sierra de Guatemala.
6. *Brackenridgia bridgesi* (Van Name, 1942): a sowbug (isopod crustacean) from the El Abra and Sierra de Guatemala.
7. *Hoplobunus boneti* (Goodnight & Goodnight, 1942): harvestman (Opiliones), known from 15 caves.
8. *Anelpistina* (*Neonicoletia*) *quinterensis* (Paclt, 1979): thysanuran or silverfish (Nicoletiidae, Zygentoma, Insecta), known from many caves in the Sierra de El Abra and Sierra de Guatemala
9. *Astyanax mexicanus/jordani*: Characiformes, Characidae cavefish, actually named *A. mexicanus/jordani* complex with *A. mexicanus* (De Filippi, 1853), *A. jordani* (Hubbs & Innes, 1936) from La Cueva Chica, *A. antrobius* (Alvarez, 1946) and *A. hubbsi* (Alvarez, 1947) from La Cueva de El Pachón and La Cueva de Los Sabinos. Thirty-three populations of *Astyanax* cavefish are currently known.

A tenth species should be included: *Spelaeomysis quinterensis* (Villalobos, 1951) (Crustacea, Mysida). While Elliott describes this species as present in Sótano del Arroyo and Cueva La Tinaja, but absent in Los Sabinos, we have found this mysid to be quite abundant in that cave, which is no surprise given that the three caves form a single hydrological system.

Materials and methods

We have visited la Cueva de Los Sabinos during five different field trips in 2013, 2017, 2019, 2022 and 2023. These expeditions were focused on *Astyanax* cavefish biology and ecology, under the auspices of permits delivered by SEMARNAT to POG and SR (02241/13; 05389/17; 1893/19; 03334/22). The 2023 visit to Los Sabinos was also under supervision of the CONANP and the local "ejido" Los Sabinos community. All our visits to this cave occurred during the dry season, in February and March (see figures for exact dates).

No samples were collected nor manipulated. Only photographs (aerial and underwater) were taken, using an Olympus TG-4 camera. All photographs shown below were taken in the Los Sabinos pool 2. This pool is a final sump of one branch of the cave, about 400 meters from and 62 meters below the entrance (Fig. 1B). The size of sponges was estimated by comparing to adult *Astyanax* cavefish, which are approximatively 6 cm long in that pool (Blin et al. 2020).

In addition, the temperature of the water in Los Sabinos pool 1 and pool 2 was recorded in March 2011, March 2017, March 2018, March 2019 and February 2022 and 2023 with a Combo Hanna HI98129. Oxygen concentrations were measured with a Hanna oxymeter HI98193.

Results and discussion

La Cueva de Los Sabinos is located in the Sierra de El Abra, SLP, Mexico (Fig. 1A). It is inhabited by a population of *Astyanax* cavefish first described as *Anoptichthys hubbsi* (Alvarez 1947), now named *Astyanax mexicanus/jordani* species complex. Inside the cave, cavefish are distributed in two distinct pools (Fig. 1B). These two pools may sometimes merge in case of important increase in water level and cave flooding, but we never witnessed this during our trips.

In February 2022, white spots on the brown stones on the bottom of pool 2 caught our attention (Fig. 1B, photo inset). Their size varied from ~2 to ~12 cm and they were clearly distinguishable. The cave isopod *Speocirolana pelaezi* and the cavefish *Astyanax mexicanus/jordani* were observed very close to some sponge specimens, but no direct interactions were seen (Fig. 1B, thin brown arrows). In February 2023, high magnification pictures were obtained and confirmed that the macroscopic aspect of the organism corresponds to typical sponge anatomy. Typical specimens between ~2 and ~7 cm on a small rock are shown in Fig. 2. Small and larger holes were observed, probably
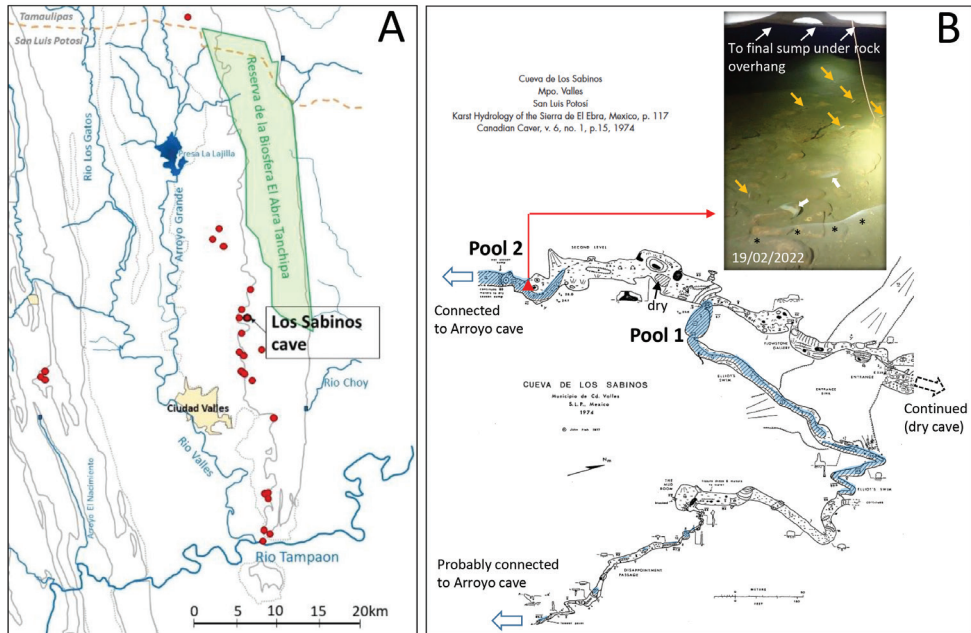
Figure 1. Maps of the geographical area and la Cueva de Los Sabinos **A** map of the Sierra de El Abra region in North East Mexico for localization of la Cueva de Los Sabinos. Red dots indicate cave locations where *Astyanax* cavefish populations are described. The Reserva de la Biosfera Sierra del Abra Tanchipa is highlighted in green **B** map of Los Sabinos topography. Pool 1 and pool 2 are identified. The red triangle points to the place where the sponges were observed in pool 2. Water bodies are colored in blue. There are more pools with the potential of also having sponges after "Elliott's swim". The large arrows show the probable connection with Sótano del Arroyo. The photo inset shows pool 2, where the first observations of the sponge were made in February 2022. Thick white arrows point to white sponges, brown arrows point to *Astyanax* cavefish populating the pond. Black asterisks indicate an artefactual line of reflection of the rocky overhang on the water surface. The small cord is an attachment for a temperature probe. Sponges are associated and clinging to the rocks scattered on the bottom of this pool.

corresponding to water intake pores and water release pores (osculum), respectively. Small spicules pointing out from the surface of the animals were also visible. We propose that these organisms are a *bona fide*, novel, non-pigmented, cave-adapted and freshwater sponge species. No genus or species name could be attributed because we did not collect samples for further anatomical or genetic analyses. However, according to macroscopic aspects, the species may belong to the genus *Racekiela* (Demospongiae, Spongillidae).

After this discovery, older pictures and underwater videos taken in Los Sabinos pool 2 during previous expeditions were re-scanned and re-examined for the presence of the sponge (Fig. 3). The earliest available evidence of the sponge presence in our photographic records dates from March 2013 (Fig. 3B). Large, recognizable non-pigmented sponges were repeatedly seen in an aerial video capture from March 2017 (Fig. 3A) and an underwater video from March 2019 (Fig. 3C). Thus, the presence of sponges in Los Sabinos pool 2 has been continual for at least 10 years (2013–2023), suggesting that they constitute an integral part of the underground community.
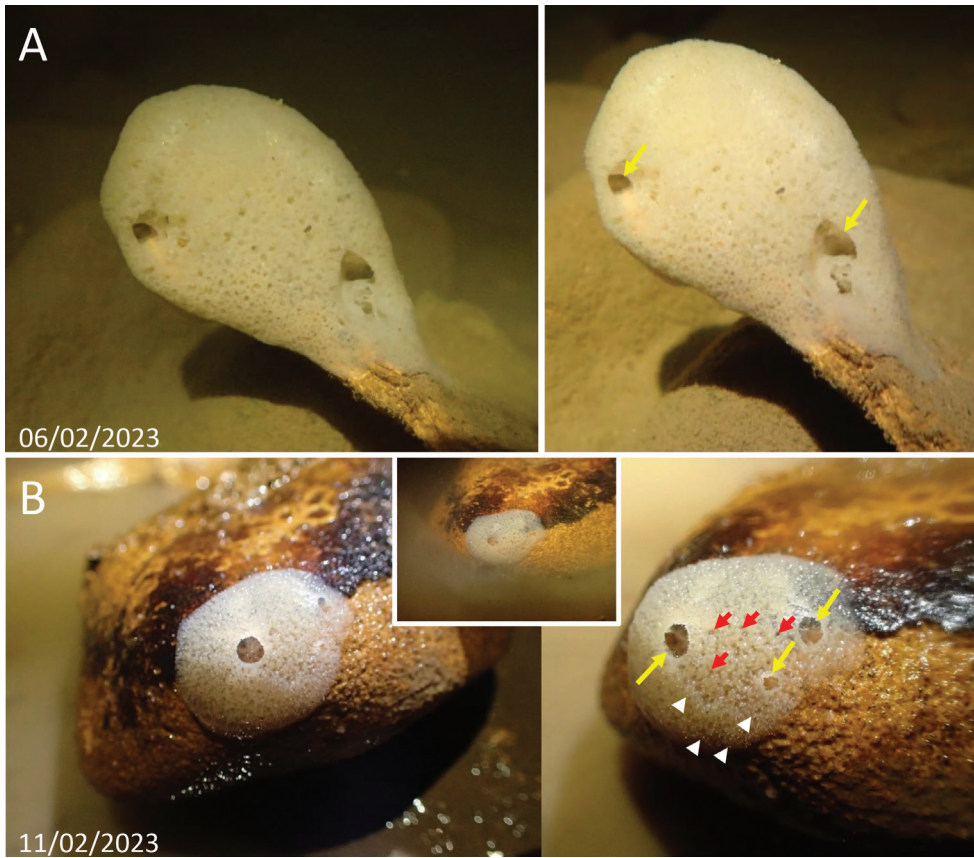
Figure 2. La Cueva de Los Sabinos pool 2, high magnification pictures, February 2023. Two specimens are shown (**A, B**). Arrows indicate water intake (red) and release (yellow) pores (osculum) as well as spicules (white). Dates of observation of the two specimens are indicated.

As of now, no sponge specimen has been observed in Los Sabinos pool 1. Yet, this pool needs to be further checked to confirm the absence of sponges. As mentioned above, the two pools are not connected during the dry season, but the two water bodies may join during exceptionally heavy rainy seasons. Interestingly, water parameters recorded in pool 1 and pool 2 were very different and may explain why the sponge is found only in the second pool (Table 1). The water temperature in the two pools differs by an average of 1.8 °C, with pool 2 always being warmer than pool 1 over the years. The minimum difference between the two pools was 1.6 °C, and the maximum difference was 2.2 °C. The dissolved oxygen concentrations were also repeatedly higher in pool 2 than in pool 1, in both February 2022 and February 2023. Studies have shown that water quality influences the distribution of sponge species (Evans 2016). Thus, the Los Sabinos sponge may have a temperature preference and/or oxygen preference that restricts its presence to pool 2, but further exploration is needed to support this preference.
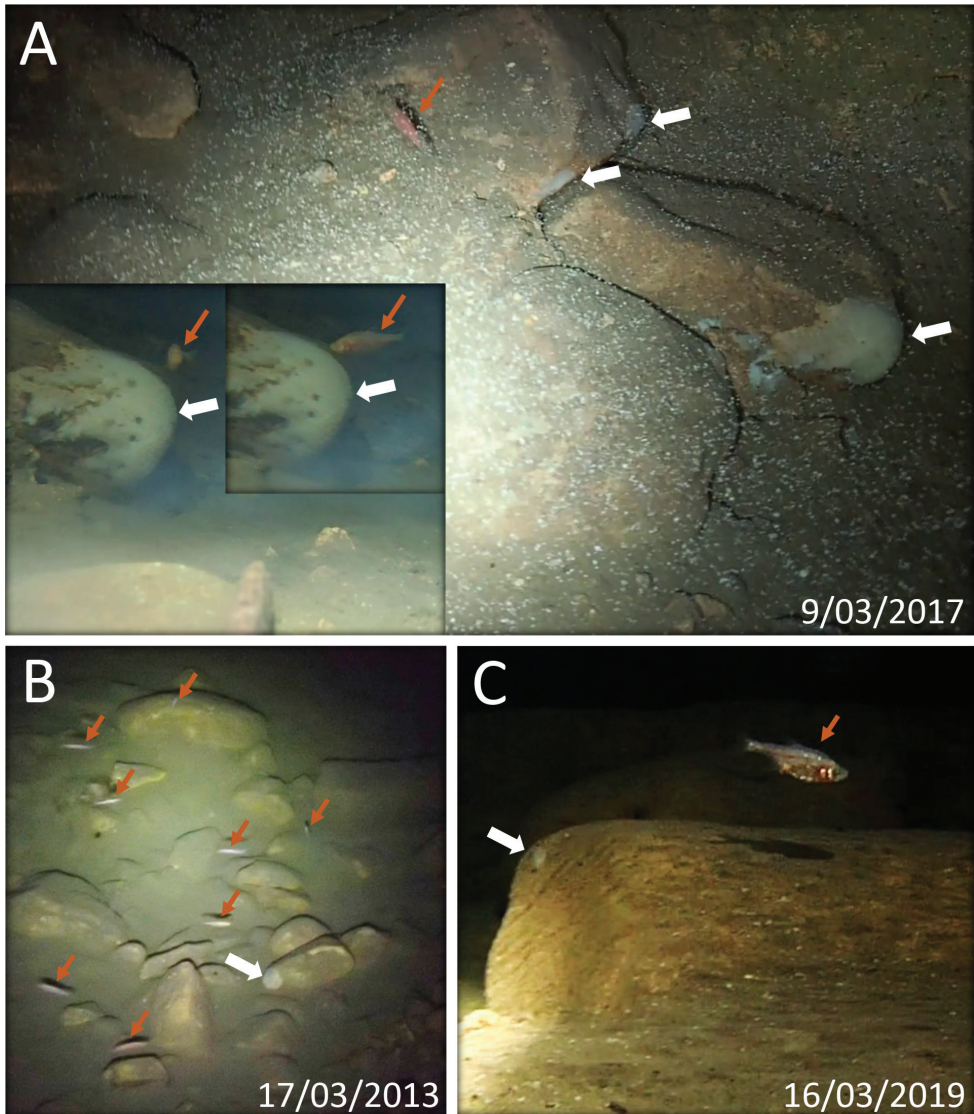
Figure 3. La Cueva de Los Sabinos pool 2. Photographs and videos showing the presence of sponges across multiple years **A–C** show photographs or snapshots from videos where sponges can be observed. Dates of observations are indicated. The continued presence of sponges throughout 2013 to 2023 suggests sponges are not accidental, but instead constitute an integral part of the underground community. Thick white arrows point to sponges, thin brown arrows point to *Astyanax* cavefish.

Another distinguishing factor for the possible exclusion of sponges from pool 1 is the substrate. The floor of pool 1 primarily consists of deep mud, where anchoring may be difficult for the sponges. While pool 2 also has large amounts of mud, sponges were systematically perched on top of the many rocks that can be found in this pool (Bell et al. 2015; Schönberg 2016).

Table 1. Physico-chemical parameters of the water in pool 1 and pool 2 are shown, at the indicated dates.

Date	O$_2$ (mg/mL)/saturation (%)		Temperature (°C)				
	Feb. 2022	Feb. 2023	March 2017	Feb. 2018	March 2019	Feb. 2022	Feb. 2023
Pool 1 (no sponge)	3.66 / 42.6%	3.72 / 42.8%	22.8	22.4	22.6	22.3	22.5
Pool 2 (with sponge)	5.73 / 70.3%	5.7 / 69.2%	24.4	24.2	24.3	24.5	24.4
Difference	2.07	1.98	1.6	1.8	1.7	2.2	1.9

Conclusion and perspectives

Excluding anchialine and brackish caves related to the sea (Gómez and Calderón-Gutiérrez 2020), this is the first record of a strictly continental and freshwater cave sponge in Mexico and North America. To our knowledge, it is only the fourth species of true freshwater cave sponge in the world. It is also the eleventh troglobitic species of la Cueva de Los Sabinos. The discovery of this non-pigmented cave sponge was unexpected. This cave is very famous within the international research community interested in the *Astyanax* cavefish model, and tourists and cavers visit it as well. The cave was first explored in 1942 and, up to now, the presence of sponges has never been reported, even though sixty different animal species were described to live there or be present in this cave (Elliott 2018).

Further exploration needs to confirm the presence or absence of the cave sponge in the first pool of la Cueva de Los Sabinos and in the other small pools in continuity (see Fig. 1B). It will also be interesting to check for the presence of the sponge in the Sótano del Arroyo, which is geographically very close to Los Sabinos and might connect through the underground aquifer with the final sump of Los Sabinos pool 2, where the sponge was discovered. Perhaps the Sótano de La Roca and the Sótano de La Tinaja will have to be checked for the sponge presence as well, as they are also close to Los Sabinos and part of the same hydrologic system.

It will also be of utmost interest to provide a detailed morphological description and to perform DNA sequence analyses to describe this probable new species of subterranean freshwater cave sponge. For the moment, we were unable to characterize this sponge species further because we did not take any samples due to the lack of proper authorization from the Mexican authorities. Our SEMARNAT permit was specific for *A. mexicanus* and, thus, no collecting attempts were done. For future research, a proper species permit will be requested. Scientific work in la Cueva de Los Sabinos will also be conducted under authorization by CONANP. This cave is under strict protection and control by the Reserva de la Biosfera El Abra Tanchipa and by the council of the ejido (or village) of Los Sabinos. The discovery of a unique, novel cave-adapted and non-pigmented sponge species in Los Sabinos further illustrates the richness of the natural resources and biodiversity in the underground realm of this region of Mexico. Follow-up studies to understand its conservation status and potential need of protection will be established (Mammola et al. 2022). Long-term and precise monitoring studies are needed to better understand the ecology of this cave ecosystem.

Acknowledgements

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