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Unique and fragile diversity emerges from Brazilian caves – two new amphibious species of *Xangoniscus* Campos-Filho, Araujo & Taiti, 2014 (Oniscidea, Styloniscidae) from Serra do Ramalho karst area, state of Bahia, Brazil

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Abstract

Two new troglobitic species of *Xangoniscus* are described from two caves of Serra do Ramalho karst area, Bambuí geomorphological group, state of Bahia. *Xangoniscus lapaensis* **sp. nov.** is described from Gruna Boca da Lapa cave, and *X. loboi* **sp. nov.** from Gruna da Pingueira II cave. Both species are blind and depigmented and show amphibious habits, as observed for all species of *Xangoniscus* described until now. *Xangoniscus lapaensis* **sp. nov.** occurs in travertine pools fed by water of the upper aquifer, and *X. loboi* **sp. nov.** occurs in a small stream, an upper vadose tributary. Both species occur in fragile microhabitats. Ecological and behavioral data, conservation remarks, and IUCN conservation assessments are included to provide background data for conservation efforts in this unique karst area.

Keywords

Bambuí geomorphological group, Limestone, Terrestrial isopods, Troglobitic, Upper Aquifer

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Introduction

Terrestrial isopods (Oniscidea) are the only crustacean lineage completely adapted to the terrestrial way of life (Hornung 2011; Richardson and Araujo 2015; Taiti 2018). The group occurs in almost all kinds of terrestrial habitats, including caves (Schmalfuss 2003; Taiti 2004). Oniscidea comprises about 4,000 species distributed in more than 500 genera and 38 families (Javidkar et al. 2015, 2017; Sfenthourakis and Taiti 2015; Dimitriou et al. 2019; Campos-Filho and Taiti 2021; WoRMS 2021).

Terrestrial isopods are one of the most representative taxa in the Brazilian subterranean environments, probably due to the special environmental conditions, such as high humidity, substrate heterogeneity, and absence of specialized predators (Fernandes et al. 2016, 2019). To date, about 210 species are known from Brazil, 70 of which, belonging to the families Styloniscidae, Philosciidae, Scleropactidae, Dubioniscidae, Platyarthridae, Porcellionidae, Armadillidae, Pudeoniscidae, and Armadillidiidae, are recorded from Brazilian caves. Thirty-two taxa are considered to be troglobites (obligatory and restricted to caves), several are troglophiles (facultative cave species), and some species are possibly trogloxenes (Souza et al. 2011; Campos-Filho et al. 2014, 2015a, 2015b, 2016, 2017a, 2017b, 2018, 2019, 2020; Bastos-Pereira et al. 2017; Fernandes et al. 2018, 2019; Cardoso et al. 2020a, 2020b, 2021). The species placed into the last two categories are difficult to assign into a correct subterranean category due to the scarcity of distribution data outside caves.

Among the troglobitic representatives of the Brazilian Oniscidea, 12 species have an amphibious way of life, all in the Styloniscidae: *Chaimowiczia uai* Cardoso, Bastos-Pereira, Souza & Ferreira, 2021, *C. tatus* Cardoso, Bastos-Pereira, Souza & Ferreira, 2021, *Iuiuniscus iuiuensis* Souza, Ferreira & Senna, 2015, *Spelunconiscus castroi* Campos-Filho, Araujo & Taiti, 2014, *Xangoniscus aganju* Campos-Filho, Araujo & Taiti, 2014, *X. ceci* Cardoso, Bastos-Pereira, Souza & Ferreira, 2020, *X. dagua* Cardoso, Bastos-Pereira, Souza & Ferreira, 2020, *X. ibiracatuensis* Cardoso, Bastos-Pereira, Souza & Ferreira, 2020, *X. itacarambiensis* Bastos-Pereira, Souza & Ferreira, 2017, *X. lundi* Cardoso, Bastos-Pereira, Souza & Ferreira, 2020, *X. odara* Campos-Filho, Bichuette & Taiti, 2016, and *X. santinhoi* Cardoso, Bastos-Pereira, Souza & Ferreira, 2020.

The present work describes two new species of *Xangoniscus* from the Serra do Ramalho karst area, state of Bahia, both occurring in special microhabitats, namely the upper aquifers fed by infiltration of water in the limestone rock. Moreover, aspects of their ecology and natural history, as well as conservation remarks about this particular and unique fauna and the region of Serra do Ramalho are given.

Materials and methods

Collections and taxonomy

Specimens of *Xangoniscus* were collected by hand with the aid of tweezers and brushes. Specimens were stored in 70% and 100% ethanol. Descriptions are based on morphological characters with the use of micropreparations in Hoyer's

medium (Anderson 1954). For each new species, the diagnosis, type material, description, etymology, and remarks are given. The *habitus* images were taken with the stereomicroscope model Motic SMZ-168 and the Celestron Microcapture Pro. The photographs were prepared with Adobe Photoshop CC Lite (v. 14.2.1). The appendages were illustrated with the aid of a camera lucida mounted on a CH2 Olympus microscope. The final illustrations were prepared using the software GIMP (v. 2.8) according to the method proposed by Montesanto (2015, 2016).

Maps were produced highlighting the caves where both *Xangoniscus* species occur, as well the hydrological attributes and pressures of economic activities in the region, representing impacts to the cave fauna. The distribution map was constructed with QGIS software (v. 3.18.1) and was edited in PowerPoint Microsoft 365 (v. 2108).

The material is deposited in the scientific collection of cave fauna of the Laboratório de Estudos Subterrâneos (LES), Universidade Federal de São Carlos, São Carlos, Brazil (Curator: Maria E. Bichuette).

Ecological and behavioral study

Both populations were studied in one field trip in October 2020, during the dry-wet season transition. Specimens were sampled *ad libitum* (sensu Altmann 1974; Martin and Bateson 2007). In addition, direct observations of other ecological and behavior data were recorded including reproductive aspects, movement, and microhabitat preferences as well as course estimations of abundance and population density. Microhabitat data included cave zone, type of substrate, depth, water current, and physicochemical variables – pH and temperature.

To assess population parameters (density and size of the population), the individuals were counted along microhabitat inside caves, and measurements (such as length, width, and total area) of the occurrence sites were taken. The population parameters were calculated by dividing the number of individuals observed by area of the occurrence site. Photographs and short videos were taken to document the behavior of both species and the influence of the microhabitat on their distribution in the caves (see Supplementary Online Material).

Study area

Serra do Ramalho karst area, State of Bahia, northeastern Brazil

The Serra do Ramalho karst area, southwestern State of Bahia, is located within the Bambuí geomorphological group, which hosts a high number of caves, mostly without legal protection. Some caves within this karst region extend for more than 15 km, developing huge cave systems (Rubbioli et al. 2019). The Serra do Ramalho karst area comprises the municipalities of Coribe, Feira da Mata, Carinhanha, and Serra do Ramalho, and plateaus are made of limestone belonging to the Bambuí geomorphologic group. Moreover, the limestone in the region forms outcrops that extend parallel to the São Francisco River (Auler et al. 2001;

Rubbioli et al. 2019). According to Köppen criteria, the region has a tropical dry climate (=semiarid) ("Aw"), with annual precipitation of about 640 mm (Beck et al. 2018, 2020). Subterranean waters are mainly exploited for human consumption (Belda et al. 2014; Rubbioli et al. 2019). Surrounding vegetation consists of "Caatinga" composed of mesophytic and xeromorphic forests interspersed with "Cerrado" (savannah-like vegetation). The two new species described herein occur in two caves of the Serra do Ramalho karst area within the São Francisco River basin (Figs 1–3). Figures 2 and 3 show details of the habitat of the new *Xangoniscus* species. Human pressures related to common economic activities in the region (mining and land use), and typical vegetation are shown in Fig. 1. Currently, mining and land use pressures represent a great threat to habitat of cave species in the region.

Results

Family Styloniscidae Vandel, 1952

Genus *Xangoniscus* Campos-Filho, Araujo & Taiti, 2014

Type species. *Xangoniscus aganju* Campos-Filho, Araujo & Taiti, 2014, by original designation.

Xangoniscus lapaensis Campos-Filho, Gallo & Bichuette, sp. nov.

http://zoobank.org/855DC7C6-D196-4B19-8340-18DB358A7277
Figures 1, 2, 4–7

Type locality. Bahia, Feira da Mata, Gruna Boca da Lapa cave (13°56'46.4"S, 44°11'12.0"W).

Type material. ***Holotype*** male (LES 27734), Bahia, Feira da Mata, Gruna Boca da Lapa cave, 15 October 2020, leg. ME Bichuette, DF Torres, JS Gallo, L Senna-Horta & JE Gallão. ***Paratypes*** 1 male (parts in slides), 2 females (LES 27735); 1 male, 3 females (one female with parts in slides) (LES 27736), same data as holotype.

Description. Body length: ♂ 8.0 mm, ♀ 9.3 mm. Body unpigmented, slightly robust, lateral sides slightly convex (Figs 2C, D, 4A–C). Dorsal surface smooth with scattered fringed scale setae (Fig. 5A). Pereonite 1 epimera with distal corners slightly developed frontwards, not surpassing cephalon, posterior corners right-angled; pereonite 2–4 epimera gradually directed backwards, posterior corners progressively more acute; pleon slightly narrower than pereon, pleonites 3–5 epimera well-developed and falciform (Figs 4B, 5C). Cephalon (Figs 4C, 5B) with large quadrangular antennary lobes; vertex with lateral depression to fit antennae when extended backwards, profrons with V-shaped suprantennal line; eyes absent. Telson (Fig. 5C) with well pronounced concave sides and broadly rounded apex. Antennula (Fig. 5D) of three articles, medial and distal articles subequal in length, second article bearing one long seta on inner margin, distal article with two long apical aesthetascs. Antenna (Fig. 5E) with fifth article of peduncle longer than flagellum; flagellum of three articles, first article longest, distal

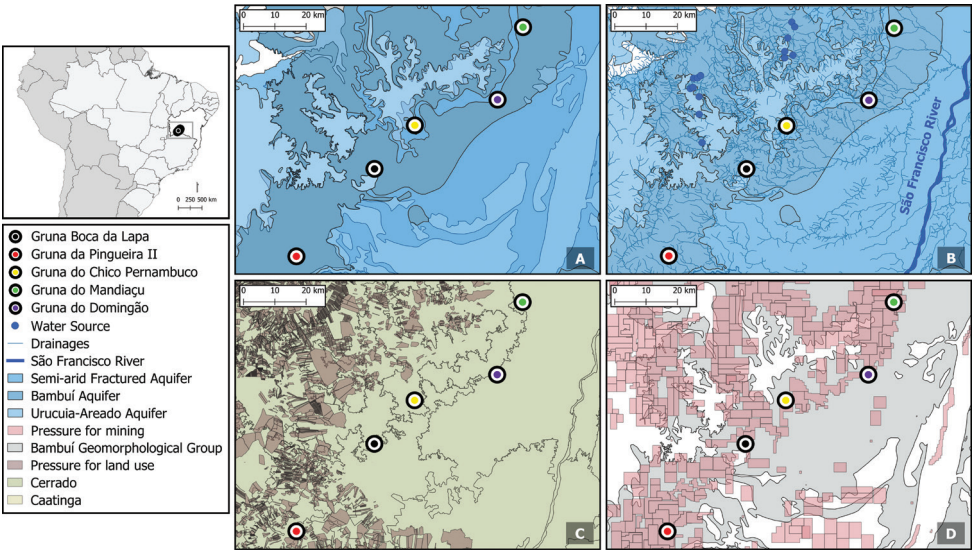

Figure 1. Maps of Serra do Ramalho karst area, State of Bahia, northeastern Brazil **A** limestone outcrops of the Bambuí geomorphological group, Fraturado and Urucuia-Areado aquifer systems showing the type-localities (caves) of *X. lapaensis* sp. nov. and *X. loboi* sp. nov. **B** headwaters and São Francisco river basin **C** pressure for land use showing human-caused alterations **D** pressure for mining activities.

article bearing tuft of free sensilla. Left mandible (Fig. 5F) with two penicils, right mandible (Fig. 5G) with one penicil and lacinia mobilis leaf-shaped with rounded apex. Maxillula (Fig. 5H) outer branch with 5 + 5 teeth, apically entire, and two thick plumose stalks; inner branch with three penicils, proximal one longer than two apical. Maxilla (Fig. 5I) with setose and bilobate apex, outer lobe smaller. Maxilliped (Fig. 5J) basis enlarged on distal portion bearing fringe of fine setae; first article of palp with two setae, distal articles fused bearing distal tufts of several thin setae; endite rectangular, outer and medial margins setose, apically with two triangular teeth and large rounded penicil. Uropod (Fig. 6A) branches inserted at same level, endopod slightly longer than exopod. Pereopods 1–7 gradually elongated, pereopods 1–7 merus, carpus, and propodus bearing sparse setae and fringed scales (discernible over 200× magnification) on sternal margin, pereopod 7 basis with scales of water conduction system; dactylus of one claw bearing many setae on inner and outer margins.

Male. Pereopods 1–6 (Fig. 6B–G) merus with proximal portion bearing fringed scales and thin setae on sternal margin. Pereopod 1 (Fig. 6B) carpus with large antennal grooming brush. Pereopod 5 (Fig. 6F) slightly depressed on distal sternal margin. Pereopod 6 (Fig. 6G) ischium flattened on sternal portion bearing brush of fine setae. Pereopod 7 (Fig. 6H) ischium with straight sternal margin, merus and carpus with no distinct modifications. Genital papilla (Fig. 7A) lanceolate, enlarged on median portion, apical part narrow. Pleopod 1 (Fig. 7B) exopod subtriangular bearing two short setae; endopod longer than exopod, narrow basal article and flagelliform distal article; basipod with triangular medial part fringed with fine and long setae on outer and

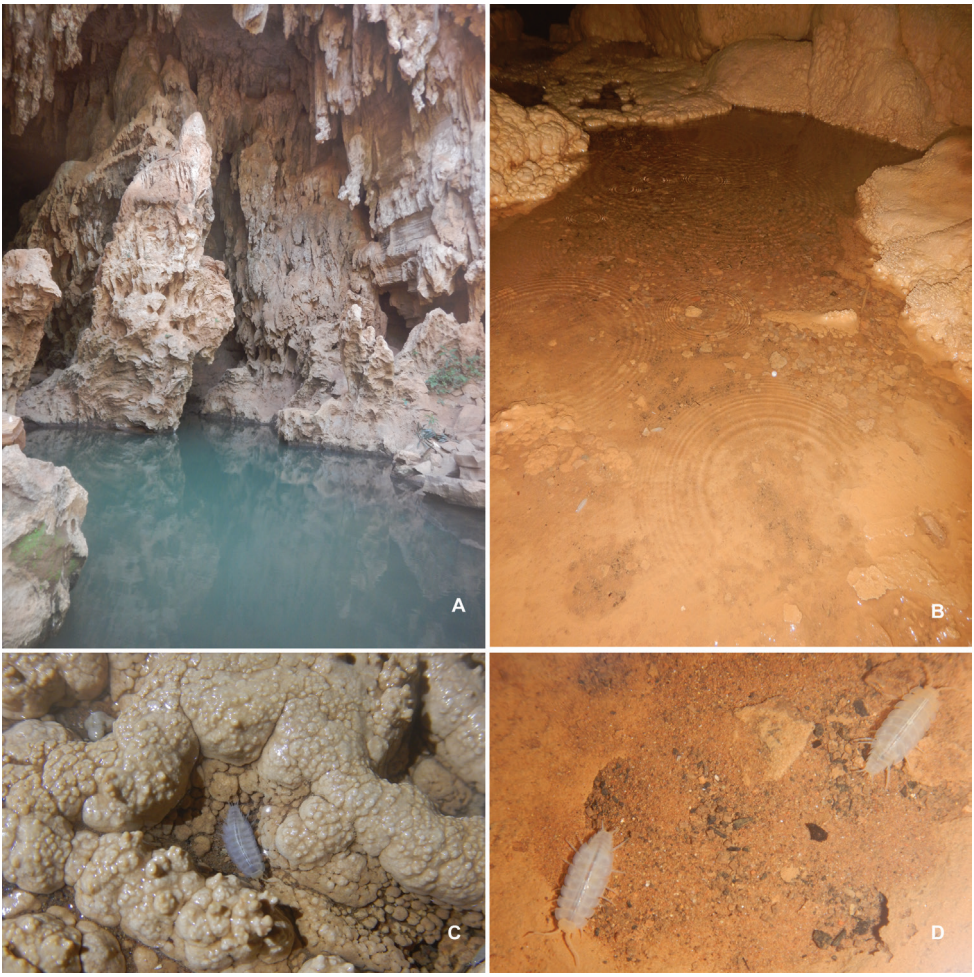

Figure 2. Natural habitat and habitus of *Xangoniscus lapaensis* sp. nov. **A** entrance of Gruna Boca da Lapa cave **B** travertine pools where *Xangoniscus lapaensis* sp. nov. occurs **C** specimen of *Xangoniscus lapaensis* sp. nov. submerged on rocky substrate at the travertine pools **D** specimens of *Xangoniscus lapaensis* sp. nov. submerged on silt and rocky substrate.

distal margins, distal margin strongly sinuous. Pleopod 2 (Fig. 7C) exopod triangular, median portion narrower, distal margin almost straight bearing three setae; endopod of two thickset articles, second article about three times as long as first, with complex apical part: distal margin subquadrangular with narrow transverse process, subapical triangular lobe strongly folded. Pleopod 3 exopod (Fig. 7D) subtriangular, longer than wide, covering pleopods 1 and 2, bearing many short setae, distal external margin straight. Pleopod 4 and 5 exopods (Fig. 7E, F) subrectangular, wider than long, with distal portion bearing many short setae.

Etymology. The new species is named after the locality where the specimens were collected, Gruna Boca da Lapa, an important cave discovered and mapped by Grupo

Bambuí de Pesquisas Espeleológicas (GBPE) in 1991 and only now is it studied in relation to its cave fauna.

Remarks. *Xangoniscus lapaensis* sp. nov. resembles *X. aganju* in the shape of the male pleopod 2 endopod; however, it differs in the vertex of the cephalon that lacks a frontal groove, and with a more pronounced suprantennal line that surpasses the antennule insertion (not surpassing in *X. aganju*). Also, the male pereopod 6 carpus lacks any modification (strongly modified in *X. aganju*, see Campos-Filho et al. 2014); the male pleopod 1 exopod is not elongated, and the male pleopod 2 exopod is narrower on inner portion. Moreover, *X. lapaensis* sp. nov. differs in the uropod endopod slightly longer than exopod (opposite in *X. aganju*) and the compact triangular shape of the male pleopod 1 exopod (elongated in *X. aganju*).

Ecology and behavior. *Xangoniscus lapaensis* sp. nov. occurs in travertine pools fed by infiltration of water through the limestone rock, dripping from the cave ceiling, and in isolated pools along the cave (Fig. 2B, C), distributed from the twilight until the aphotic zone, something that demonstrates that its distribution is related to pools formed by infiltration water, i.e., its special habitat is available. The microhabitat is composed of rocky substrate, with little silt and sand sediment. The water is clear, with a very slow current, and a depth varying from 0.05 m to 0.15 m (Fig. 2B–D). Physicochemical variables were typical of karst aquifers that have high pH values: pH = 8.5–9.0, T = 23.5 °C, very different from the conditions in the base-level river (pH = 7.5, T = 25 °C). A small quantity of organic and particulate matter was observed in the small pools. Population density in the pools was high, ranging 6.5 to 32 individuals.m^{-2} and the abundance was 74 individuals in an area of 2.8 m^2. The locomotor behavior was very particular, exhibiting fast and effective walking movements when submerged. However, when emerging from water, individuals showed a slow walking behavior on terrestrial substrate (see Supplementary Online Material 1). *Xangoniscus lapaensis* sp. nov. did not show photophobic behavior (indifferent to flashlight of about 600 lumens), and also seemed to exhibit intraspecific interactions, with no agonistic behavior. Adults and juveniles were observed in the pools, representing possible recruitment at the end of the dry season. It appears that *X. lapaensis* sp. nov. occurs preferentially in microhabitats fed by infiltration water, since no individual was recorded in the stream of the cave, a base-level river. This is very specific and unique, and probably the main habitat of this species is the spaces in the rocks filled by water.

Xangoniscus loboi **Campos-Filho, Gallão & Bichuette, sp. nov.**
http://zoobank.org/0572EC0F-C9B4-4B05-804C-3606AC59A8AF
Figures 1, 3, 8–11

Type locality. Bahia, Feira da Mata, Gruna da Pingueira II cave (14°12'04.3"S, 44°24'39.1"W).

Type material. ***Holotype*** male (LES 27737), Bahia, Feira da Mata, Gruna da Pingueira II cave, 14 October 2020, leg. ME Bichuette, DF Torres, JS Gallo & JE Gallão. ***Para-***

Figure 3. Natural habitat and habitus of *Xangoniscus loboi* sp. nov. **A** karst area where Gruna da Pingueira II cave is located **B** karst and entrance of Gruna da Pingueira II cave **C** specimen of *Xangoniscus loboi* sp. nov. submerged on the silt substrate **D** damn inside the cave **E** specimens of *Xangoniscus loboi* sp. nov. feeding on seed fruits **F** specimens of *Xangoniscus loboi* sp. nov. submerged on silt and rocky substrate **G** specimens of *Xangoniscus loboi* sp. nov. submerged on the rocky substrate **H** stream inside Gruna da Pingueira II cave where *Xangoniscus loboi* sp. nov. occurs.

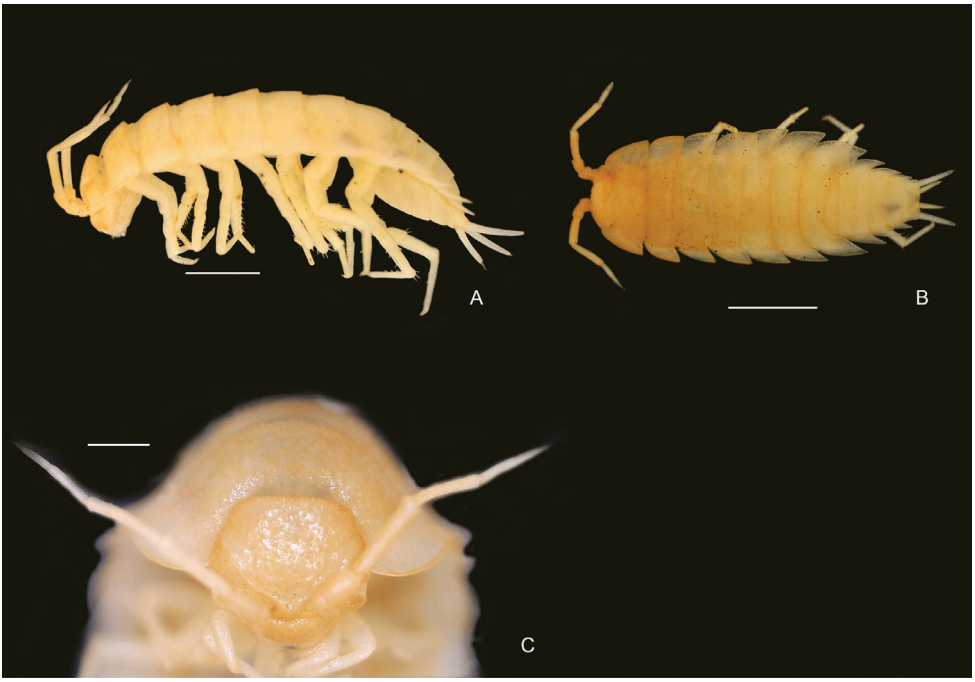

Figure 4. *Xangoniscus lapaensis* sp. nov. **A** habitus, lateral view **B** habitus, dorsal view **C** cephalon and pereonites 1–4, dorsal view. Scale bar: 2 mm (**A, B**); 1 mm (**C**).

types 2 males, 3 females (one female with parts in micropreparations) (LES 27738), 6 males (one male in micropreparations), 4 females (LES 27739), same data as holotype.

Description. Body length: ♂ 12 mm, ♀ 10 mm. Body unpigmented, slender and elongated, lateral sides almost parallel (Figs 3C, E, 8A–C). Dorsal surface smooth with scattered fringed scale setae (Figs 8C, 9A). Pereonite 1 epimera with distal corners not developed frontwards, posterior corners right-angled; pereonites 2–4 epimera gradually directed backwards, posterior corners progressively more acute; pleon slightly narrower than pereon, pleonites 3–5 epimera very short, slightly visible on dorsal view (Figs 8A, 9C). Cephalon (Figs 8D, 9B) with large quadrangular antennary lobes; vertex with slightly lateral depression to fit antennae when extended backwards, profrons with V-shaped suprantennal line, not surpassing antennule insertion; eyes absent. Telson (Fig. 9C) with well pronounced concave sides and broadly rounded apex. Antennula (Fig. 9D) of three articles, proximal and distal articles subequal in length, second article bearing short setae on inner margin, distal article with five aesthetascs. Antenna (Fig. 9E) with fifth article of peduncle longer than flagellum; flagellum of four articles, first article longest, distal article bearing tuft of free sensilla. Left mandible (Fig. 9F) with two penicils, right mandible (Fig. 9G) with one penicil and lacinia mobilis leaf shaped. Maxillula (Fig. 9H) outer branch with 5 + 5 teeth, apically entire, and two thick plumose stalks; inner branch with three penicils, proximal one longer than two apical. Maxilla (Fig. 9I) with setose and bilobate apex, outer lobe smaller bearing one strong

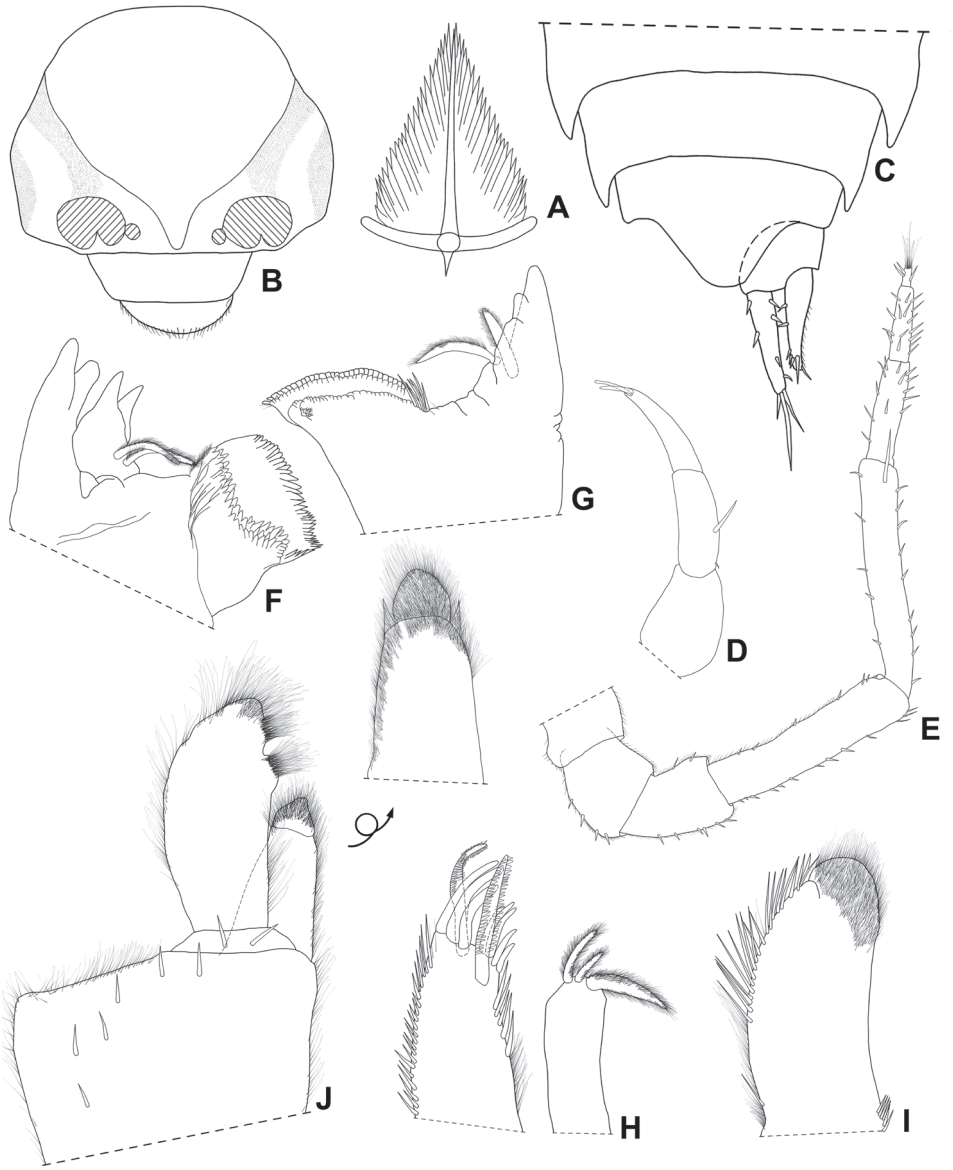

Figure 5. *Xangoniscus lapaensis* sp. nov. Male **A** dorsal scale-setae **C** pleonites 4 and 5, telson and uropod. Female **B** cephalon, frontal view **D** antennula **E** antenna **F** left mandible **G** right mandible **H** maxillula **I** maxilla **J** maxilliped.

seta. Maxilliped (Fig. 9J) basis enlarged on distal portion bearing fringe of fine setae; first article of palp with two setae, distal articles fused bearing distal tufts of fine setae; endite rectangular, outer and medial margins setose, apically with one triangular teeth and large triangular penicil. Uropod (Fig. 10A) branches inserted at same level, endo-

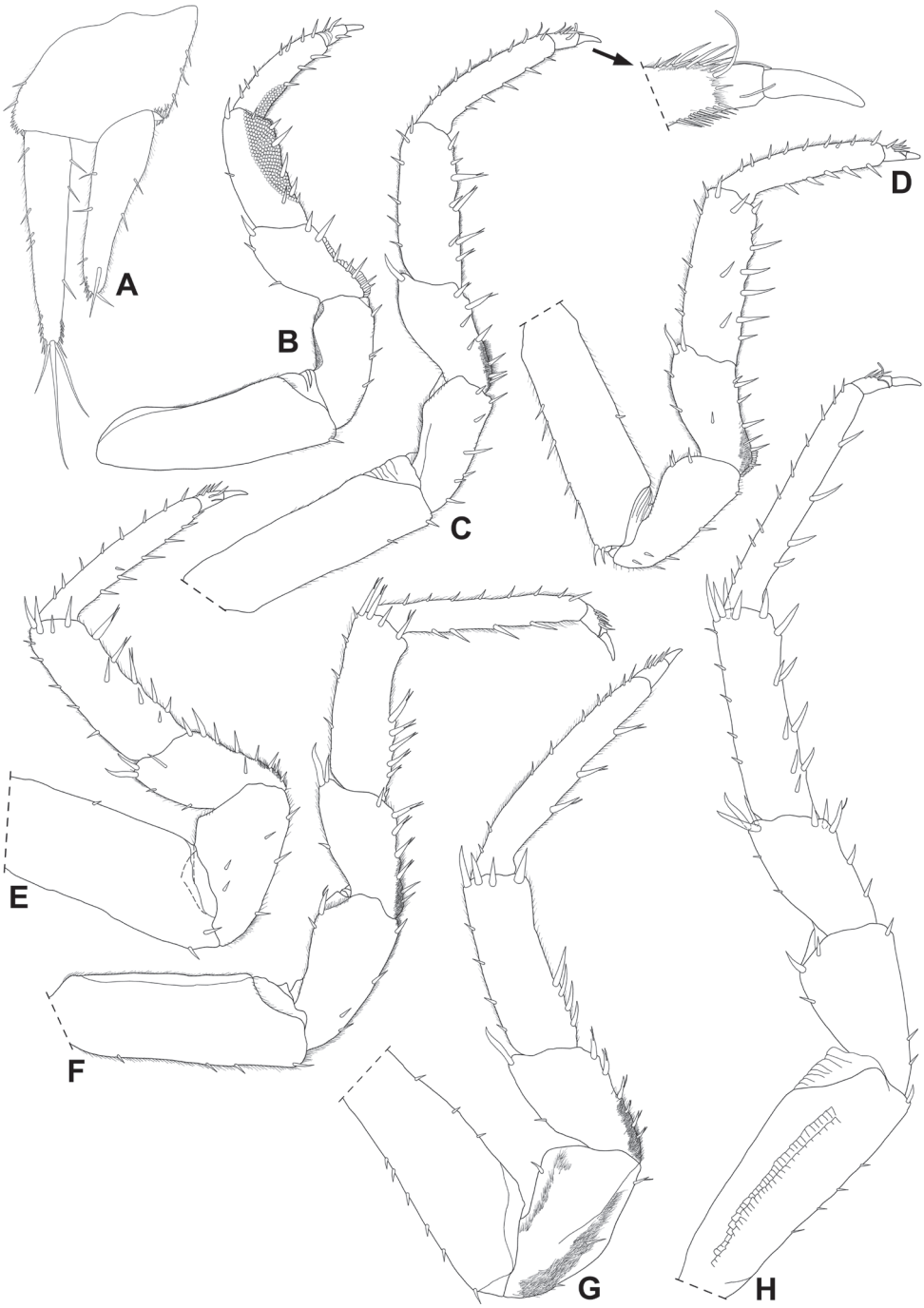

Figure 6. *Xangoniscus lapaensis* sp. nov. Male **A** uropod **B** pereopod 1 **C** pereopod 2 **D** pereopod 3 **E** pereopod 4 **F** pereopod 5 **G** pereopod 6 **H** pereopod 7.

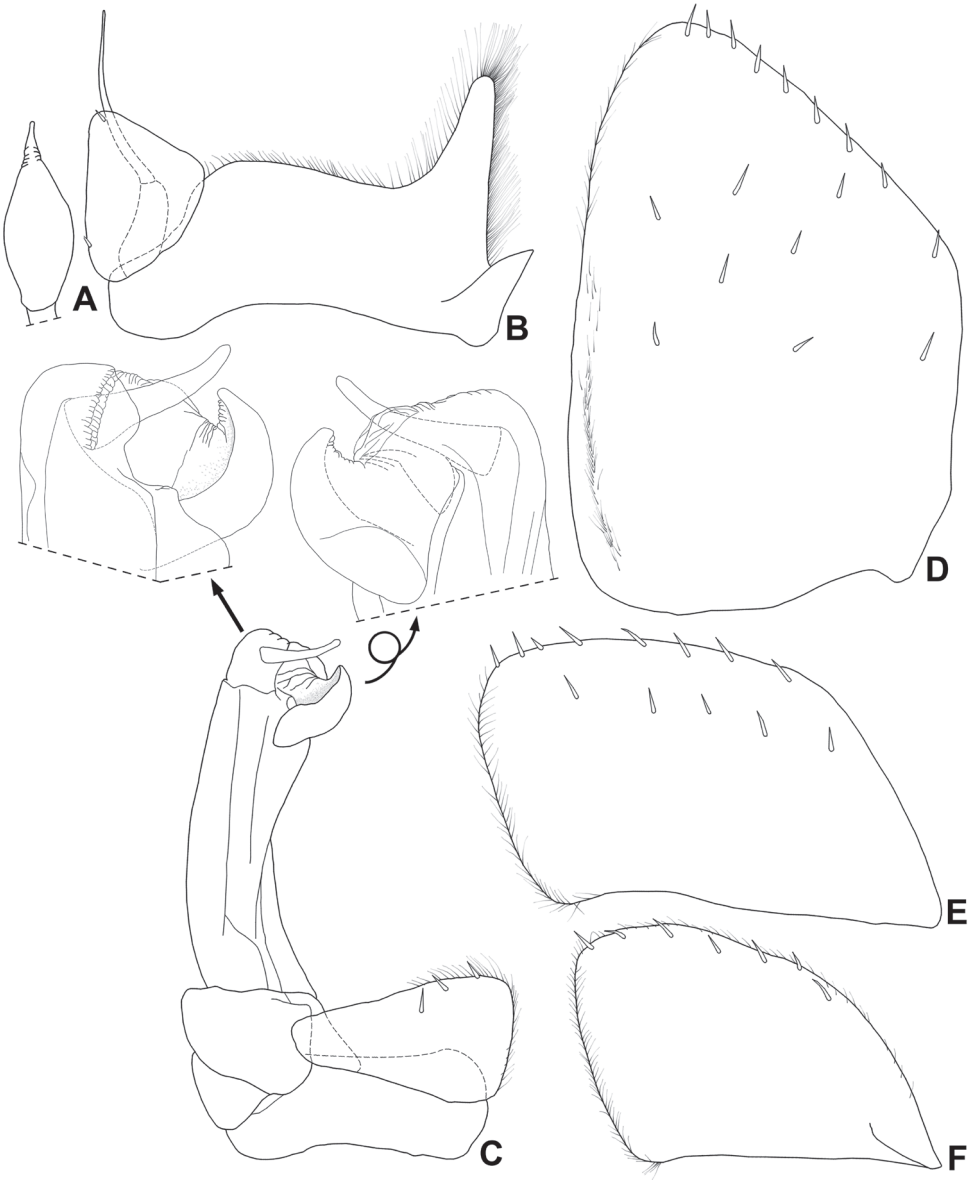

Figure 7. *Xangoniscus lapaensis* sp. nov. Male **A** genital papilla **B** pleopod 1 **C** Pleopod 2 **D** pleopod 3 exopod **E** pleopod 4 exopod **F** pleopod 5 exopod.

pod and exopod subequal in length. Pereopods 1–7 gradually elongated, pereopods 1–7 merus, carpus and propodus bearing sparse setae and fringed scales (discernible over 200× magnification) on sternal margin, pereopod 7 basis with scales of water conduction system; dactylus of one claw bearing many setae on inner and outer margins.

Male. Pereopods 1–3 (Fig. 10B–D) merus with proximal lobe bearing fringed scales and fine setae on sternal margin. Pereopods 4–6 (Fig. 10E-G) merus with dense field of

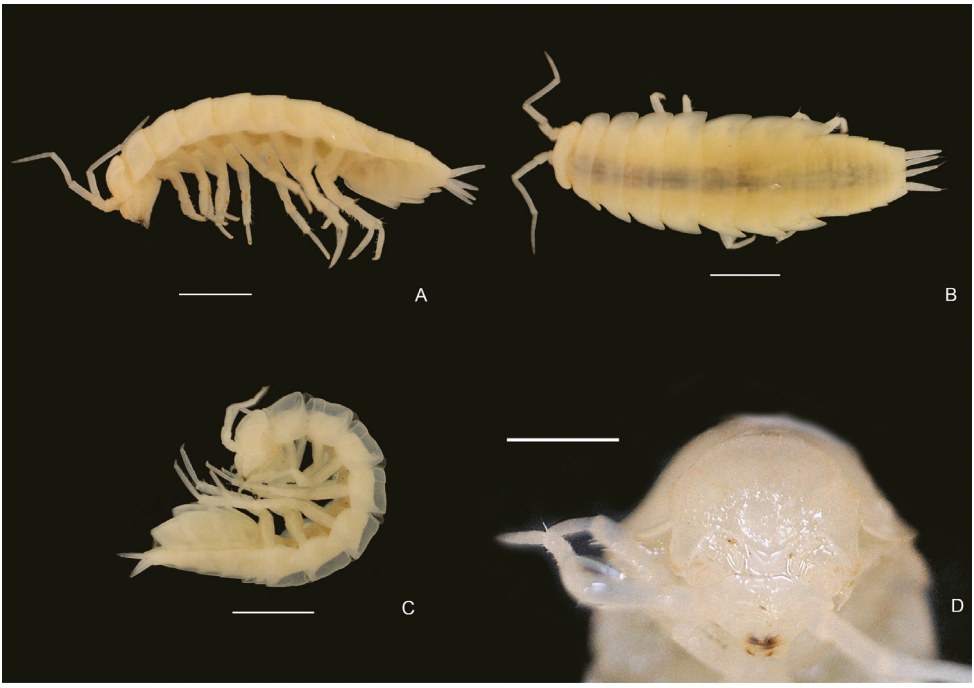

Figure 8. *Xangoniscus loboi* sp. nov. **A** habitus, lateral view **B** habitus, dorsal view **C** habitus, lateral view **D** cephalon and pereonite 1, frontal view. Scale bar: 2 mm (**A-C**); 1 mm (**D**).

short setae on sternal margin. Pereopod 6 (Fig. 10G) merus bearing brush of setae on sternal margin. Pereopod 7 (Fig. 10H) ischium straight on sternal margin, merus, and carpus with no distinct modifications. Genital papilla (Fig. 11A) lanceolate, enlarged on median portion, apical part narrow and pointed. Pleopod 1 (Fig. 11B) exopod subtriangular, longer than wide, inner and outer margins bearing fringe of fine setae; endopod slightly longer than exopod, narrow basal article and flagelliform distal article; basipod with stout and broadly rounded medial part bearing fringe of fine setae along outer and distal margins. Pleopod 2 (Fig. 11C) exopod triangular, median portion narrower, distal margin almost straight bearing three setae; endopod of two thickset articles, second article about three times as long as first, with complex apical part: distal margin rounded with two narrow transverse processes, subapical circular lobe strongly folded. Pleopod 3 exopod (Fig. 11D) triangular, slightly longer than wide, covering pleopods 1 and 2, bearing many short setae on distal margin. Pleopod 4 exopod (Fig. 11E) subrectangular, wider than long, distal portion bearing many short setae. Pleopod 5 exopod (Fig. 11F) subtrapezoidal, distal margin rounded bearing many short setae.

Etymology. The new species is named after Alexandre Lobo for all his contributions for knowledge and conservation of the Brazilian speleological heritage.

Remarks. *Xangoniscus loboi* sp. nov. differs from all other species of the genus in having the proximal lobe of male pereopods 1–3 merus covered with scales and fine setae, and in the shape of the distal complex apparatus of the male pleopod 2 endopod.

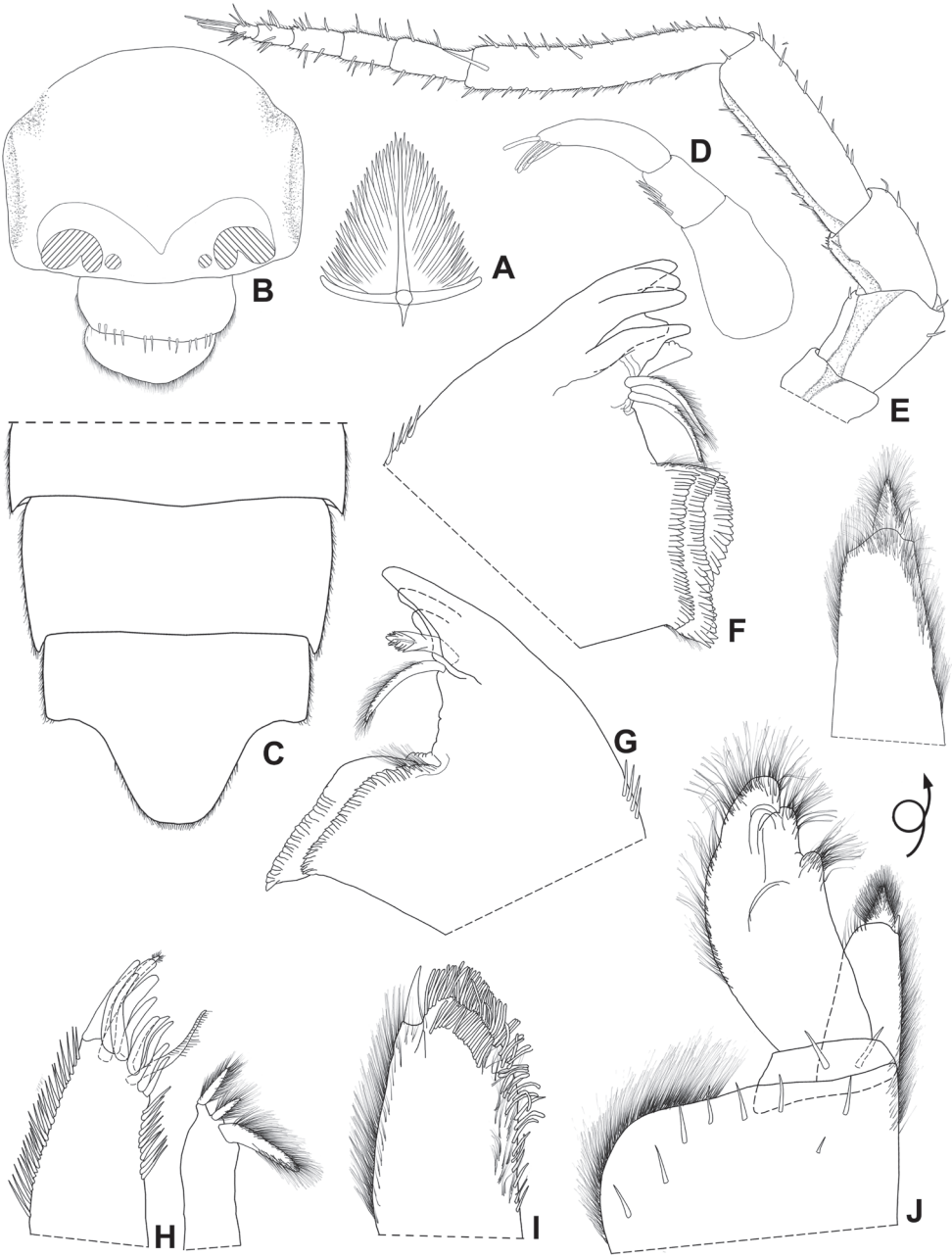

Figure 9. *Xangoniscus loboi* sp. nov. Male **A** dorsal scale-seta **B** cephalon, frontal view **C** pleonites 4, 5 and telson **D** antennula **E** antenna **F** left mandible **G** right mandible **H** maxillula **I** maxilla **J** maxilliped.

Ecology and behavior. *Xangoniscus loboi* sp. nov. occurs in a small stream formed by infiltration water in the limestone rock (Fig. 3). The small stream crosses an extension of about 9 m through the cave entrance and the twilight zones, with

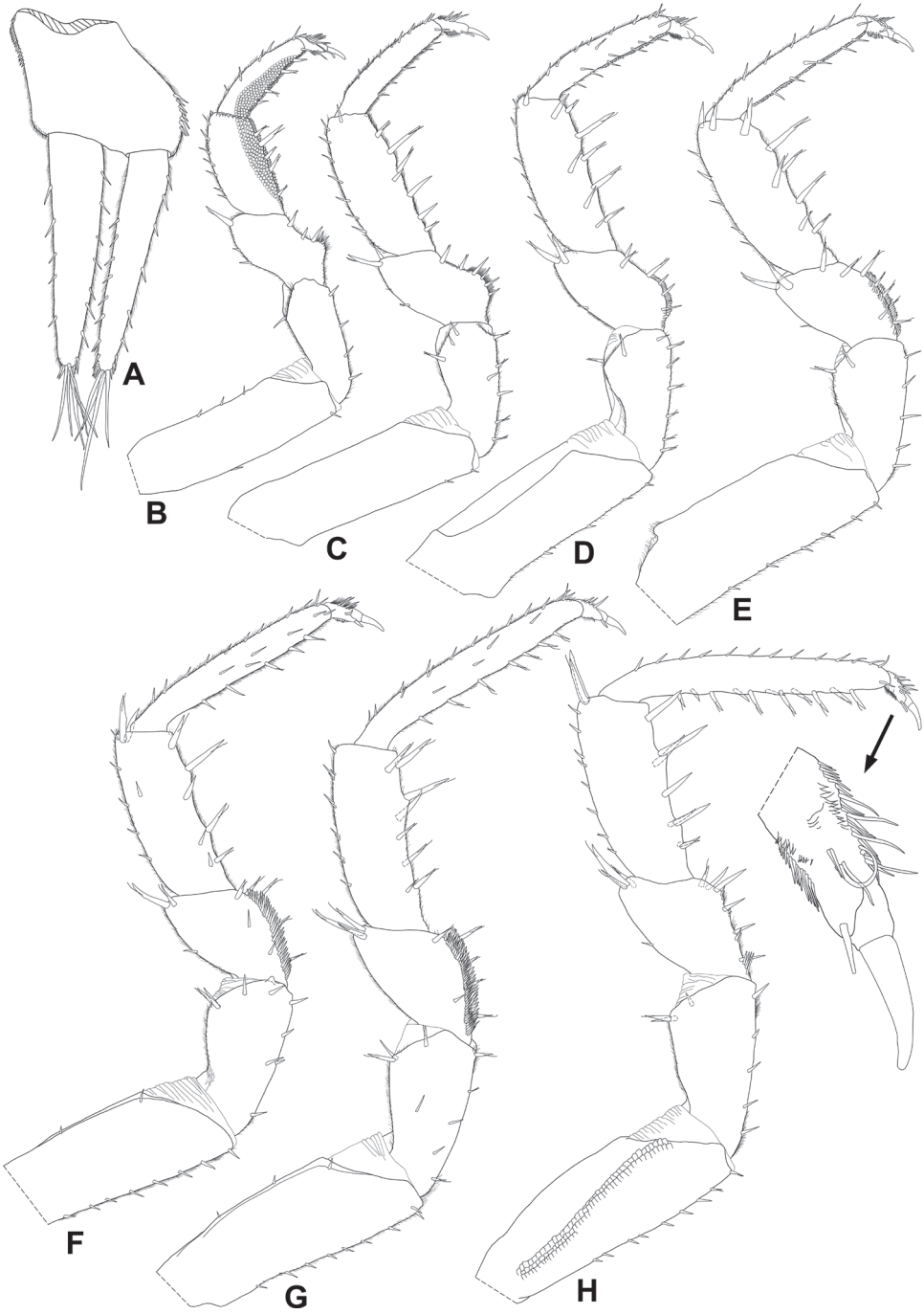

Figure 10. *Xangoniscus loboi* sp. nov. Male **A** uropod **B** pereopod 1 **C** pereopod 2 **D** pereopod 3 **E** pereopod 4 **F** pereopod 5 **G** pereopod 6 **H** pereopod 7.

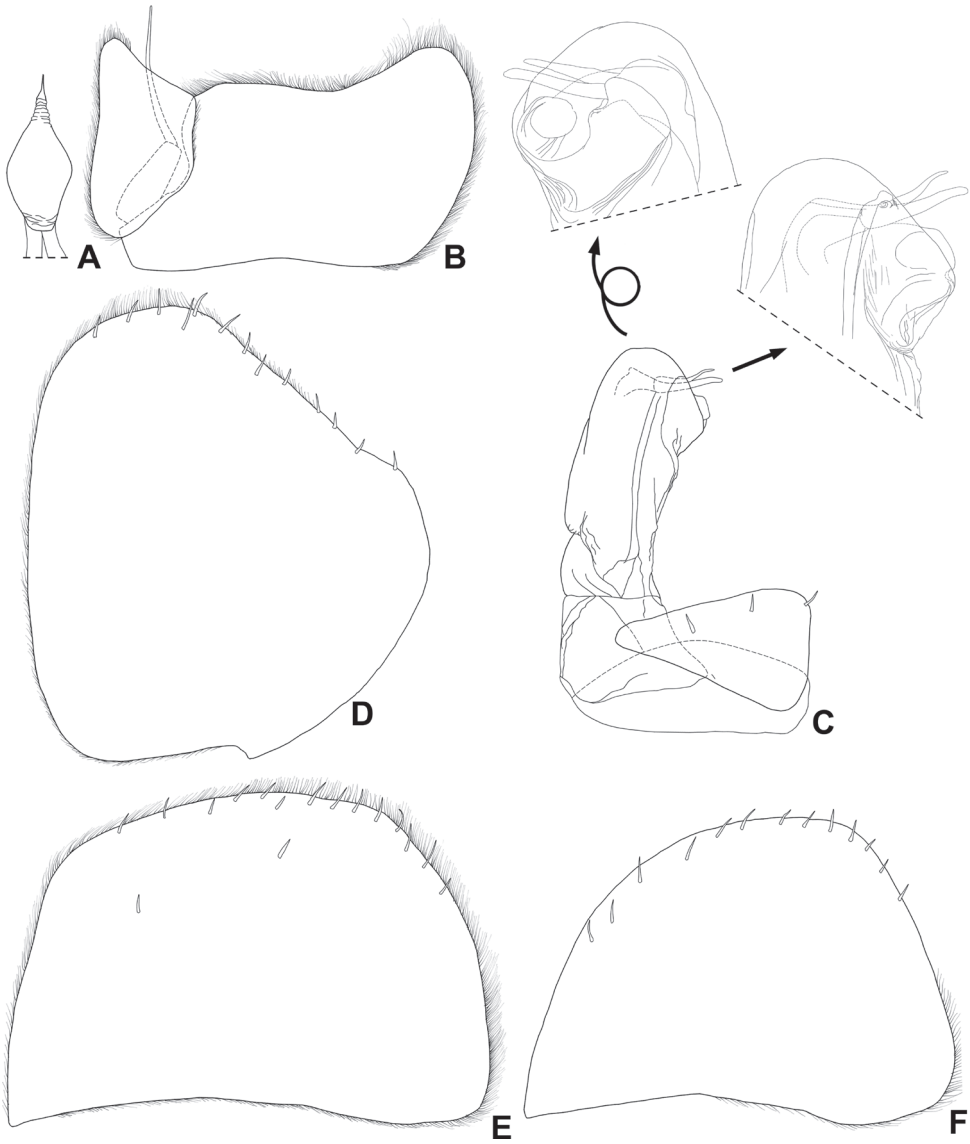

Figure 11. *Xangoniscus loboi* sp. nov. Male **A** genital papilla **B** pleopod 1 **C** Pleopod 2 **D** pleopod 3 exopod **E** pleopod 4 exopod **F** Pleopod 5 exopod.

width varying from 0.2 to 1.2 m and depth from 0.05 to 0.25 m. Along the stream, two small waterfalls (ca. 0.6 m of length) with a rocky substrate were observed. In these stretches, the individuals were seen constantly climbing the substrate (see Supplementary Online Material 2). The bottom of the stream is composed mainly of rocky substrate (concretions and small pebbles), with silt and sand sediment in the bottom, offering rich shelter for the isopods (Fig. 3 C, E–H). The water is clear, with a very slow current. Physicochemical variables are typical of karst aquifers (high

pH values), pH = 8.0–8.5, T = 24.1 °C. A low load of organic and particulate matter was observed in the stream, with some seeds of fruits in the sector close to the cave entrance. The population density in three sectors of the stream were: 54 inds.m^{-2}, 67 inds.m^{-2} and 342 inds.m^{-2}; minimal abundance was 455 individuals in an area of ca. 5.0 m^2. As observed for *X. lapaensis* sp. nov., the locomotor behavior was very particular. *Xangoniscus loboi* sp. nov. shows an efficient walking behavior when submerged (see Supplementary Online Material 3) and climbs vertical stretches (upstream direction) in the stretches with relatively strong water flow. They are clumsy out of water (they do not perform as well as in the submersion behavior), but they adhere very well on dry rock. We also observed copulation: three different couples in reproductive behavior, mated for several minutes (at least 5 min.) (see Supplementary Online Material 4 and 5). Several adults and juvenile individuals were observed along the stream, representing possible recruitment at the end of the dry season. Other interesting behaviors are the indifference to light, even under strong flashlight (ca. 600 lumens), and the interactions among several individuals, touching each other, showing no signs of agonistic behavior. In relation to feeding behavior, some individuals were observed foraging on vegetal debris and seeds (see Supplementary Online Material 6). It appears that *X. loboi* sp. nov. occurs preferentially in microhabitats fed by infiltration water in the limestone. This is very specific and unique, and probably the main habitat of this species is the spaces in rocks filled by water.

Discussion

Both species described herein show the classical signs of troglomorphism (eyes absent and body depigmented), as observed for the other eight valid species of *Xangoniscus*, i.e., *X. aganju*, *X. ceci*, *X. dagua*, *X. ibiracatuensis*, *X. itacarambiensis*, *X. lundi*, *X. odara* and *X. santinhoi*. This could be a pattern for the entire genus, since all species described until now show these same characteristics. Morphological variability in these two characters was not observed for any species of *Xangoniscus*. To date, no *Xangoniscus* species has been recorded outside caves, in the epigean environment, which may denote an ancient isolation of this group.

Morphologically, *X. lapaensis* sp. nov. and *X. aganju* (including specimens from Gruna Chico Pernambuco and Domingão caves, see Campos-Filho et al. 2019) exhibit some level of similarities in the pereopods 5 and 6 of males, e.g., merus and carpus with fringed scales and dense field of thin setae on external margin and carpus distally depressed (see figures of *X. aganju* in Campos-Filho et al. 2014). The complex shape of the distal portion of the male pleopod 2 endopod is quite similar among specimens of *X. aganju* from Gruna do Mandiaçu cave (type locality) and Gruna Chico Pernambuco cave and *X. lapaensis* sp nov. (see figure 13C in Campos-Filho et al. 2014, and figure 7B in Campos-Filho et al. 2019), and specimens of *X. aganju* from Domingão cave and *X. loboi* sp. nov. shares the presence of two transverse processes (see figure 7C in Campos-Filho et al. 2019). Taking into account the geographic distribution of these

species (see Fig. 1) and these morphological characteristics, it is likely that these species represent a species complex. Future phylogenetic analyses potentially will elucidate the hypotheses proposed herein.

The Serra do Ramalho region has been locally exploited historically by agriculture activities and charcoal production on a small scale. At a broader scale, mining projects and large agriculture production along the boundaries of the region pose future threats (Gallão & Bichuette, 2018) (Fig. 1C, D). To date, no effective law guarantees protection of the caves in the region. However, both Gruna Boca da Lapa and Gruna da Pingueira II caves are type-localities of endemic and probably rare troglobites, classifying these caves as maximum relevance *sensu* Brazilian legislation (BRASIL 2008). However, this action likely will not be sufficient, because protection is restricted to a small portion of an extensive massif. The creation of a conservation unit for the entire karst area would probably be the most effective action to ensure the preservation of the biological and speleological heritage of the Serra do Ramalho karst region.

A preliminary evaluation of the conservation status of these species was conducted following the IUCN (International Union of Conservation of Nature) classification. Despite their high population density and abundance, *Xangoniscus lapaensis* sp. nov. and *Xangoniscus loboi* sp. nov. were classified as Critically Endangered (CR) according to the criteria B2ab(iii). Both species are endemic, restricted to their type-locality and found in a specific microhabitat (travertine pools and streams formed by infiltration water). The surrounding areas of the cavities are impacted by deforestation for pastures and the native vegetation is present only at their entrances (Fig. 3A, B). Furthermore, the Gruna Boca da Lapa cave is threatened by uncontrolled tourism. These threats, combined with the impacts mentioned above for the Serra do Ramalho region, justify the conservation status proposed in this evaluation.

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Supplementary online material

1. Video on YouTube. *Xangoniscus lapaensis* sp. nov. locomotor behavior in the Gruna da Lapa. https://www.youtube.com/watch?v=jFZXOb7BAdg
2. Video on YouTube. *Xangoniscus loboi* sp. nov. moving against the water flow in a small waterfall. https://www.youtube.com/watch?v=ZOwsvT_9gjA
3. Video on YouTube. *Xangoniscus loboi* sp. nov. locomotor behavior in a travertine pool on Gruna da Pingueira II. https://www.youtube.com/watch?v=iuGvySw4SV0
4. Video on YouTube. *Xangoniscus loboi* sp. nov., copula. https://www.youtube.com/watch?v=u2_AK_PfMHs
5. Video on YouTube. *Xangoniscus loboi* sp. nov., copula. https://www.youtube.com/watch?v=yL1PVQHz7OY
6. Video on YouTube. *Xangoniscus loboi* sp. nov., foraging and interacting behaviour. A https://www.youtube.com/watch?v=xQxKHrb80kM

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RESEARCH ARTICLE

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Observations on the habitat and feeding behaviour of the hypogean genus *Eukoenenia* (Palpigradi, Eukoeneniidae) in the Western Italian Alps

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Abstract

The order Palpigradi includes species characterized by millimetric size and a characteristic flagellum with bristles at the end of the opisthosoma. They represent one of the less well-known and obscure arachnid orders. In this paper, observations were made on the ecology and feeding behavior of species belonging to the genus *Eukoenenia* Börner, 1901, from the Western Italian Alps. Direct observations and photographic documentation of 141 individuals in their cave habitat, allowed the recording of data on the physical and trophic conditions such as the presence/absence of trophic resources, temperature and relative humidity, of the underground environment in which they were found. Results showed that the species of this taxon are not as rare as previously reported and that their presence is mainly influenced by temperature, relative humidity, trophic resources and the presence of two speleothems: rimstone dams and rafts. The combination of our observations as well as data previously published highlights that the taxon can have predatory and saprophagous feeding behavior depending on the availability of the food resources. This work represents the starting point for a further investigation of the taxon.

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Keywords
Arachnida, microwhip scorpions, subterranean biology, caves

Introduction

Palpigradi is the last described arachnid order and, to date, one of the lesser known and studied. The order includes small size species characterized by the whip-like flagellum with bristles at the end of the opisthosoma. The world fauna of living palpigrads consists of more than 100 described species divided in two families: Eukoeneniidae and Prokoeneniidae (Harvey 2002; Giribet et al. 2014).

The first scientific investigation of the species in this order began in 1885 in Sicily (Italy), where the zoologist Giovanni Battista Grassi (Grassi and Calandruccio 1885) discovered a "mysterious species" of arachnid that he described as *Koenenia mirabilis* Grassi & Calandruccio, 1885, now *Eukoenenia mirabilis* (Grassi & Calandruccio, 1885). Silvestri (1905) described for the first time the anatomy of the species and he was followed by other authors but many aspects of the biology of the order, including food selection and reproduction, are still unknown (Condé 1996; Smrž et al. 2013; Christian et al. 2014; Parimuchová et al. 2021). Molecular phylogeny of the taxon was performed only by Giribet et al. (2014) that demonstrated the monophily of the order and of the family of Eukoeneniidae.

The elective habitats of the species in the genus *Eukoenenia* are interstitials (Howarth 1983; Mammola 2019) and the species can be ecologically classified in soil- and cave-dwelling (Mammola et al. 2021b). The cave-dwelling species have been reported walking on the cave walls and ground (Condé 1996), speleothems (Souza and Ferreira 2010), decomposing wood (Balestra et al. 2019), sand banks near water stream (Souza et al. 2020) and on the surface of water pools (Christian et al. 2014; Balestra et al. 2019).

There is currently little known about the feeding of palpigrades. An observation of *Eukoenenia* hunting springtail provides evidence of a predatory habit (Lukić 2012), however, the presence of Cyanobacteria in their gut indicates an alternative food source (Smrž et al. 2013) and scavenging is also another possibility. A recent study based on molecular analysis (NGS) on the gut-content of *Eukoenenia spelaea* (Peyerimhoff, 1902) in Ardovská Cave, Slovakia, supports carnivory behaviour (Parimuchová et al. 2021).

Because there is minimal data available about the biology of the species of this genus, a continuous survey was carried out within 20 caves in Piedmont and Liguria regions (Northern Italy) in order to describe and better understand some aspects of their biology: nutrition, microhabitat and distribution. In particular, the following questions have been addressed: i) Is the paucity of records of this taxon related with bias in sampling or is it related with the low density of the populations? ii) What kind of environmental/ecological parameters affect the presence and the distribution of palpigrades in caves? iii) What is the trophic role of the taxon (potential predatory species, saprophagous species or both)?

Materials and methods

Study area

South Western Italian Alps are rich in natural caves of diverse origins and at different altitudes. The range of cave temperature is varied, with cold caves yielding temperatures lower than 8 °C, mainly at high altitude, and more mesophilic caves with average temperature at around 9–13 °C. This area is also rich in artificial cavities of interest for hypogean life study (Fig. 1).

Figure 1. Location of the sampling (maps used for the plate retrieved from https://d-maps.com/carte.php?num_car=2232&lang=en, https://d-maps.com/carte.php?num_car=5894&lang=en, https://d-maps.com/carte.php?num_car=8273&lang=en and modified).

In this area, six hypogean species of the *E. spelaea* species-complex have been reported (Balestra et al. 2019; Christian et al. 2014; Isaia et al. 2011; Pantini and Isaia 2019): *E. spelaea*, *E. strinatii* Condé, 1977, *E. bonadonai* (Condé, 1979), *E. roscia* Christian, 2014, *E. lanai* Christian, 2014, and an undescribed species of *Eukoenenia*. (E. Christian in litteris).

The investigated cavities are listed in Table1 and reported in Fig. 1. Their entrances are located at altitudes between 554 and 2163 m a.s.l. Two of them, Grotta della Mottera and Carsena di Piaggia Bella, are cold caves while the others 18 have an average temperature ranging between 9 and 13 °C. Miniera superiore di Monfieis is an artificial cavity.

The majority of the data reported in this paper were collected from the Bossea cave, the first Italian show cave, that is composed by different environments, and from four other cavities where *Eukoenenia strinatii* have been already reported (Balestra et al. 2019).

Sampling and observation method

Pre-evaluations based on literature research, biogeographic and field observations allowed the selection of 20 different caves where biotic and abiotic factors that can influence palpigrade life as temperature (T °C), relative humidity (RH%), light intensity (LI lux), type of substrate, speleothems, presence, and abundance of decomposing organic matter and presence of water and fractures were monitored.

Random observations of each cave were performed in different periods of the year, with a minimum number of 2 days per cave in two different seasons. One up to six observation areas were defined for each cave at different distances from the cave entrance (ranging from few meters from the entrance to great depths) depending on the cave structure and the presence of peculiar microhabitat (Table 1). These areas were coded according to the cave name (three letters) and a number, corresponding with the inner sampling sites. The observations for each cave area lasted 20 minutes. Visual encountered surveys, supported with the acquisition of macrophotographs were performed. This is a non-invasive method, however, it required a priori biological knowledge of the taxa observed for the identification and it does not allow always a correct determination at a specific level of the taxa. Palpigrades are tiny arachnids and often close related species have very uniform general morphology, consequently, specimens for species identification were collected manually, using a metal spatula made by one of the authors (E.L.) which is more effective in collecting palpigrades, and placed directly in 70–96% ethyl alcohol in sampling tubes. Identification was performed by specialists, as listed in the acknowledgments.

Macrophotography has several advantages, such as highlighting details not visible at naked eye or reviewing behaviour of the observed individuals, however, also different disadvantages, especially in cave where bringing photographic equipment can be complex due to habitat impediments (see Balestra et al. 2021; Mammola et al. 2021a). Photographs of the specimens were taken using a Canon EOS 550D and 760D reflex cameras equipped with MP-E 65 mm Macro lenses and MT-24EX Macro flash and Canon EOS 70D reflex camera equipped with EF 100 mm Macro lens 1:2.8 USM

Table 1. Examined caves and sampling areas. The identification code of the cave in the Piedmont and Ligurian cadastre caves is shown in the column Id.

Cavity name	Cavity type	Id	Geographical coordinates WGS84	Municipality/ area	Alps	Altitude (m a.s.l.)	Linear cave development (m)	Geology	Sampling areas	Distance from the entrance (m)
Buco del Partigiano	Cave	PI1315	44.506897 N, 7.2932269 E	Roccabruna	Cottian	1170	13	Dora-Maira units	PAR1	0–50
Buranco di Bardineto	Cave	LI364–1364	44.1994345 N, 8.12489 E	Bardineto	Liguri	770	2060	Limestones and dolostones of the Brianzonesi units	BUR1	100–200
									BUR2	100–200
									BUR3	100–200
									BUR4	200–300
Grotta occidentale del Bandito	Cave	PI1003	44.290002 N, 7.427431 E	Valdieri	Marittime	714	690	Sub-Brianzonesi units	BAN1	0–50
Grotta di Bossea	Cave	PI108	44.241548 N, 7.8398498 E	Frabosa Soprana	Ligurian	836	2800	Marbles and dolomitic marbles of the Brianzonesi Units	BOS1	400–500
									BOS2	500–600
									BOS3	500–600
									BOS4	500–600
									BOS5	400–500
									BOS6	400–500
									BOS7	200–300
Grotta Rio dei Corvi	Cave	PI884	44.3012409 N, 7.9939506 E	Lisio	Ligurian	800	300	Piedmontese zone	RDC1	100–200
Grotta del Baraccone	Cave	PI309	44.2733643 N, 8.0867885 E	Bagnasco	Ligurian	1040	39	Dolomites of the Piemontesi Units	BAR1	0–50
Grotta di Rio Borgosozzo	Cave	PI695	44.1219794 N, 7.8294941 E	Ormea	Ligurian	870	90	Limestones and dolostones of the Brianzonesi units	RBS1	0–50
									RBS2	0–50
									RBS3	0–50
Grotta dei Dossi	Cave	PI106	44.3404645 N, 7.7429119 E	Villanova Mondovì	Ligurian	626	580	Dolomites of the Piemontesi Units	DOS1	0–50
									DOS2	0–50
									DOS3	0–50
Grotta "Barôn Litrôn"	Cave	PI1214	44.2616808 N, 7.4093819 E	Valdieri	Marittime	1050	861	Limestones of the Subbrianzonesi Units	BAL1	100–200
Grotta del Caudano	Cave	PI121–122	44.2930025 N, 7.7905788 E	Frabosa Sottana	Ligurian	780	3200	Marbles and dolomitic marbles of the Brianzonesi Units	CAU1	400–500
									CAU2	500–600
Grotta delle Vene	Cave	PI103	44.1514836 N, 7.7513324 E	Upega	Ligurian	1558	6285	Limestones and dolostones of the Brianzonesi units	VEN1	500–600
Grotta di Rossana	Cave	PI1010	44.5325001 N, 7.4306342 E	Rossana	Cozie	554	195	Dolostones of the Piemontesi Units	ROS1	100–200
Miniera superiore di Monfieis	Mine	CAPI7046	44,3604152 N, 7,2658630 E	Demonte	Cozie	1750	474	Brianzonese zone	MIN1	0–50

Cavity name	Cavity type	Id	Geographical coordinates WGS84	Municipality/ area	Alps	Altitude (m a.s.l.)	Linear cave development (m)	Geology	Sampling areas	Distance from the entrance (m)
Grotta della Taramburla	Cave	PI204–227–228–284	44.1177137 N, 7.9699005 E	Caprauna	Ligurian	860	2570	Limestones and dolostones of the Brianzonesi units	TAR1 TAR2 TAR3	0–50 50–100 50–100
Garb del Dighea	Cave	PI126	44.1293344 N, 7.9333236 E	Ormea	Ligurian	1590	143	Limestones and dolostones of the Brianzonesi units	DIG1 DIG2	50–100 50–100
Grotta di Costacalda	Cave	PI3613	44.2402139 N, 7.8485798 E	Roburent	Ligurian	1037	1701	Marbles and dolomitic marbles of the Brianzonesi Units	GCC1 GCC2 GCC3	400–500 0–50 200–300
Grotta dell'Orso di Ponte di Nava	Cave	PI118	44.1190364 N, 7.8753066 E	Ormea	Ligurian	810	705	Limestones and dolostones of the Brianzonesi units	PDN1 PDN2	0–50 100–200
Arma Cornarea	Cave	LI252	44.11901 N, 7.8101901 E	Piancavallo-Armasse	Ligurian	1038	120	Brianzonese-Ligurian zone	COR1	100–200
Grotta della Mottera	Cave	PI242	44.1997424 N, 7.8350138 E	Ormea	Ligurian	1325	22279	Brianzonese-Ligurian zone	MOT1	100–200
Carsena di Piaggia Bella	Cave	PI160	44.1673208 N, 7.7060822 E	Briga Alta	Marittime	2163	43000	Brianzonese-Ligurian zone	CPB1	200–300

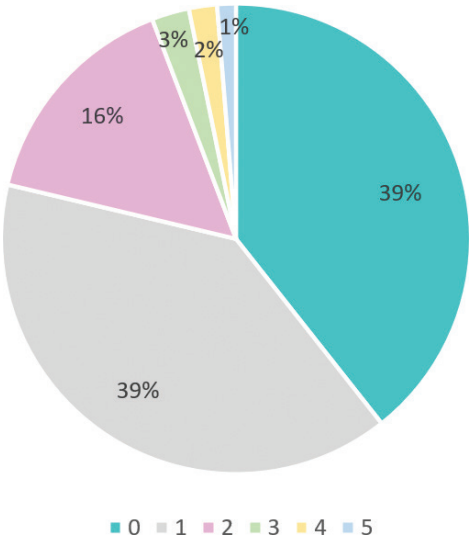

Figure 2. Number of specimens observed for sampling site.

and integrated flash. For the environmental photos, a Canon EOS 70D reflex camera equipped with EFS 18–55 mm lens and a Canon Power Shot D30 camera were used.

Environmental parameters were recorded in the presence of living animals. An HD 2101.1 Delta Ohm Thermohygrometer with a combined probe HP 472AC %RH and temperature Pt100 were used for environmental temperature and relative humidity measurements (Thermohygrometer HD 2101.1 Delta Ohm: Temperature: -200 / +650 °C, Relative humidity: 0.0 / 100.0%. HP 472AC %RH and temperature Pt100 combined probe: Area of use: -20 / +80 °C, 0 / 100% RH; accuracy: ±2% (5 to 95% RH), ±3% (95 to 99% RH), ±0.3 °C (-20 to + 80 °C)). Due to the use of a 2.5 m long extension for the termohygrometer probe, all parameters were recorded without close human presence. pH was measured using litmus paper (Vetrotecnica 08.3000.00 pH 1 / 11).

Results

Survey and records

114 surveys in caves were performed finding at least one *Eukoenenia* specimen in 65.79% of the cases. No specimens were observed in cold caves (Grotta della Mottera and Carsena di Piaggia Bella) or in Grotta occidentale del Bandito, despite a previous record from this cave (Brignoli 1976). A total of 143 individuals in 17 different caves of the Western Italian Alps were observed (Table 2). 110 individuals were observed in the last 4 years of monitoring. The number of specimens observed in a sampling site in a day ranged from 0 to 5 (Fig. 2), with an average of 0.93 individuals/sampling site/day, and the number of specimens in a cave ranged from 0 to 8 per day.

Table 2. Observational records of *Eukoenenia* species under natural conditions in the western Italian Alps caves.

Taxa	Cave	Sampling area	dd/mm/yy	Alive or dead	Find surface	Trophic resource	T (°C)	UR%	pH
Eukoenenia sp. nov.	Grotta Della Taramburla	TAR2	23/04/2017	dead	water surface	yes	–	–	–
	Grotta Della Taramburla	TAR1	23/04/2017	alive	water surface	yes	–	–	–
	Grotta Della Taramburla	TAR1	23/04/2017	alive	water surface	yes	–	–	–
	Grotta Della Taramburla	TAR1	24/12/2017	alive	water surface	yes	–	–	–
	Grotta Della Taramburla	TAR1	31/12/2017	alive	water surface	yes	11.7	100	7
	Grotta Della Taramburla	TAR1	31/12/2017	alive	speleothem	yes	11.7	100	7
	Grotta Della Taramburla	TAR3	31/12/2017	alive	water surface	yes	–	–	7
	Grotta Della Taramburla	TAR1	30/09/2018	alive	water surface	no	13.6	91.1	7
	Garb del Dighea	DIG2	11/05/2018	alive	water surface	no	9.4	89.8	7
	Garb del Dighea	DIG1	11/05/2018	dead	water surface	yes	–	–	–
Eukoenenia strinatii	Grotta Di Bossea	BOS5	20/12/2009	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS6	21/12/2009	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS5	18/08/2010	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS5	15/01/2011	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS1	11/03/2011	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS1	12/03/2011	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS1	16/08/2011	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS5	20/10/2011	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS6	21/12/2011	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS5	26/12/2011	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS5	27/01/2012	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS5	13/08/2012	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS6	20/02/2013	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS5	19/09/2013	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS5	30/04/2014	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS5	17/11/2014	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS7	29/12/2014	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS1	15/08/2015	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS5	26/06/2016	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS5	26/06/2016	alive	speleothem	yes	–	–	–
	Grotta Di Bossea	BOS6	30/08/2016	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS5	10/10/2016	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS6	10/10/2016	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS6	10/10/2016	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS1	19/10/2016	alive	near water surface	yes	–	–	–
	Grotta Di Bossea	BOS1	02/09/2017	dead	water surface	no	–	–	–
	Grotta Di Bossea	BOS1	02/09/2017	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS1	02/09/2017	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS1	13/09/2017	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS1	13/09/2017	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS5	26/09/2017	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS1	17/12/2017	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS1	17/12/2017	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS2	17/12/2017	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS2	17/12/2017	alive	near water surface	yes	–	–	–
	Grotta Di Bossea	BOS2	17/12/2017	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS2	17/12/2017	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS5	26/12/2017	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS3	09/01/2018	dead	water surface	–	–	–	–
	Grotta Di Bossea	BOS5	09/01/2018	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS3	13/01/2018	alive	water surface	yes	11.9	81.5	7
	Grotta Di Bossea	BOS1	13/01/2018	alive	water surface	yes	9.4	92.9	7
	Grotta Di Bossea	BOS3	20/01/2018	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS5	20/01/2018	alive	water surface	yes	–	–	–

Taxa	Cave	Sampling area	dd/mm/yy	Alive or dead	Find surface	Trophic resource	T (°C)	UR%	pH
Eukoenenia strinatii	Grotta Di Bossea	BOS1	03/05/2018	alive	water surface	yes	10.3	90.1	7
	Grotta Di Bossea	BOS1	27/05/2018	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS5	02/06/2018	alive	water surface	yes	9.3	96	7
	Grotta Di Bossea	BOS5	02/06/2018	alive	water surface	yes	9.3	96	7
	Grotta Di Bossea	BOS5	02/06/2018	dead	water surface	yes	9.3	96	7
	Grotta Di Bossea	BOS5	26/06/2018	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS5	26/06/2018	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS5	30/06/2018	alive	water surface	yes	9.6	92.5	7
	Grotta Di Bossea	BOS5	30/06/2018	alive	water surface	yes	10	94.1	7
	Grotta Di Bossea	BOS5	30/06/2018	alive	water surface	yes	9	94.3	7
	Grotta Di Bossea	BOS5	30/06/2018	dead	water surface	yes	9	94.3	7
	Grotta Di Bossea	BOS4	11/07/2018	alive	water surface	no	11	85.6	7
	Grotta Di Bossea	BOS5	11/07/2018	dead	water surface	–	–	–	–
	Grotta Di Bossea	BOS5	11/07/2018	alive	water surface	no	11.2	83.6	7
	Grotta Di Bossea	BOS5	11/07/2018	alive	water surface	yes	10.1	89.9	7
	Grotta Di Bossea	BOS5	11/07/2018	alive	water surface	yes	10.1	89.9	7
	Grotta Di Bossea	BOS5	11/07/2018	dead	water surface	Yes	–	–	–
	Grotta Di Bossea	BOS6	11/07/2018	alive	water surface	yes	10.2	93.4	7
	Grotta Di Bossea	BOS6	11/07/2018	alive	water surface	yes	10.2	93.4	7
	Grotta Di Bossea	BOS5	29/08/2018	alive	water surface	yes	10.5	90.4	7
	Grotta Di Bossea	BOS5	29/08/2018	alive	water surface	yes	10.5	90.4	7
	Grotta Di Bossea	BOS6	29/08/2018	alive	water surface	yes	10.1	92.2	7
	Grotta Di Bossea	BOS6	29/08/2018	dead	water surface	–	–	–	–
	Grotta Di Bossea	BOS5	18/09/2018	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS6	18/09/2018	alive	water surface	yes	–	–	–
	Grotta Di Bossea	BOS5	27/01/2019	alive	water surface	yes	9.5	94.5	7
	Grotta Di Bossea	BOS5	27/01/2019	dead	water surface	–	–	–	–
	Grotta Di Bossea	BOS6	06/10/2019	alive	near water surface	yes	9.6	92	7
	Grotta Di Bossea	BOS6	06/10/2019	dead	water surface	–	–	–	–
	Grotta Di Bossea	BOS6	06/10/2019	dead	water surface	–	–	–	–
	Grotta Di Bossea	BOS6	06/10/2019	alive	water surface	yes	9.6	92	7
	Grotta Di Bossea	BOS6	06/10/2019	alive	water surface	yes	9.6	92	7
	Grotta Di Bossea	BOS5	06/10/2019	alive	water surface	yes	9.2	95.5	7
	Grotta Di Bossea	BOS5	06/10/2019	alive	water surface	yes	9.2	95.5	7
	Grotta Di Bossea	BOS5	06/10/2019	alive	water surface	no	9.2	96.1	7
	Grotta Rio dei Corvi	RDC1	05/01/2017	alive	water surface	yes	–	–	–
	Grotta Rio dei Corvi	RDC1	05/01/2017	alive	water surface	yes	–	–	–
	Grotta Rio dei Corvi	RDC1	15/03/2017	alive	water surface	yes	–	–	–
	Grotta Rio dei Corvi	RDC1	15/03/2017	alive	water surface	yes	–	–	–
	Grotta di Rio Borgosozzo	RBS1	01/05/2017	alive	water surface	yes	–	–	–
	Grotta di Rio Borgosozzo	RBS2	03/06/2018	alive	water surface	no	10.8	88.7	7
	Grotta di Rio Borgosozzo	RBS3	03/06/2018	alive	water surface	yes	10.9	87.7	7
	Grotta di Rio Borgosozzo	RBS3	03/06/2018	alive	water surface	no	10.9	87.7	7
	Grotta Del Baraccone	BAR1	11/03/2017	alive	water surface	yes	–	–	–
	Grotta Dei Dossi	DOS1	30/12/2017	dead	water surface	–	12.2	87.3	7
	Grotta Dei Dossi	DOS2	30/12/2017	dead	water surface	–	12.3	83.8	7
	Grotta Dei Dossi	DOS3	30/12/2017	alive	water surface	yes	13.3	81	7
	Grotta Dei Dossi	DOS3	30/12/2017	alive	water surface	yes	13.3	81	7
Eukoenenia bonadonai	Grotta Delle Vene	VEN1	30/12/2016	alive	water surface	yes	–	–	–
	Grotta Delle Vene	VEN1	28/05/2017	alive	water surface	yes	–	–	–
	Grotta Del Caudano	CAU1	14/02/2010	alive	water surface	yes	–	–	–
	Grotta Del Caudano	CAU1	26/02/2012	alive	water surface	yes	–	–	–
	Grotta Del Caudano	CAU1	11/01/2017	alive	water surface	yes	–	–	–
	Grotta Del Caudano	CAU1	11/01/2017	alive	water surface	yes	–	–	–
	Grotta Del Caudano	CAU2	26/02/2017	dead	water surface	–	–	–	–
	Grotta Del Caudano	CAU2	26/02/2017	alive	water surface	yes	–	–	–
	Grotta Barôn Litrôn	BAL1	13/04/2003	alive	Wet wood	yes	–	–	–

Taxa	Cave	Sampling area	dd/mm/yy	Alive or dead	Find surface	Trophic resource	T (°C)	UR%	pH
Eukoenenia bonadonai	Grotta Barôn Litrôn	BAL1	03/12/2006	alive	water surface	no	–	–	–
	Grotta Barôn Litrôn	BAL1	17/01/2012	alive	speleothem	no	–	–	–
	Grotta Barôn Litrôn	BAL1	11/10/2016	alive	water surface	yes	–	–	–
Eukoenenia spelaea	Buco Del Partigiano	PAR1	11/09/2011	alive	under stone	no	–	–	–
	Buco Del Partigiano	PAR1	21/07/2012	alive	speleothem	no	–	–	–
	Buranco Di Bardineto	BUR1	20/05/2018	alive	water surface	yes	–	–	7
	Buranco Di Bardineto	BUR2	20/05/2018	alive	water surface	yes	–	–	7
	Buranco Di Bardineto	BUR2	20/05/2018	alive	water surface	yes	–	–	7
	Buranco Di Bardineto	BUR3	20/05/2018	alive	water surface	yes	–	–	7
	Buranco Di Bardineto	BUR3	20/05/2018	alive	water surface	yes	–	–	7
	Buranco Di Bardineto	BUR3	20/05/2018	alive	water surface	yes		–	7
	Buranco Di Bardineto	BUR3	20/05/2018	alive	water surface	yes	–	–	7
	Buranco Di Bardineto	BUR4	20/05/2018	alive	water surface	yes	–	–	6
	Buranco Di Bardineto	BUR2	13/10/2019	alive	water surface	yes	13.7	82.8	7
	Buranco Di Bardineto	BUR2	13/10/2019	alive	water surface	yes	12	88.2	7
	Buranco Di Bardineto	BUR3	13/10/2019	alive	water surface	no	12.4	86.7	7
	Buranco Di Bardineto	BUR3	13/10/2019	dead	water surface	–	–	–	–
Eukoenenia roscia	Grotta Di Rossana	ROS1	16/02/2012	alive	speleothem	no	–	–	–
	Grotta Di Rossana	ROS1	16/02/2012	alive	speleothem	no	–	–	–
	Grotta Di Rossana	ROS1	15/08/2012	alive	speleothem	no	–	–	–
	Grotta Di Rossana	ROS1	03/03/2013	alive	speleothem	no	–	–	–
Eukoenenia lanai	Miniera Sup. Di Monfieis	MIN1	12/09/2010	alive	speleothem	no	–	–	–
	Miniera Sup. Di Monfieis	MIN1	12/09/2010	alive	water surface	yes	–	–	–
	Miniera Sup. Di Monfieis	MIN1	12/09/2010	alive	speleothem	yes	–	–	–
Eukoenenia sp. 1*	Grotta Di Costacalda	GCC1	03/05/2018	alive	water surface	no	–	–	–
	Grotta Di Costacalda	GCC2	11/05/2018	alive	water surface	no	–	–	–
	Grotta Di Costacalda	GCC3	27/05/2018	alive	water surface	yes	–	–	–
Eukoenenia sp. 2**	Grotta Dell'orso Di Ponte Di Nava	PDN1	03/06/2018	alive	water surface	yes	10.9	87.6	7
	Grotta Dell'orso Di Ponte Di Nava	PDN2	03/06/2018	alive	water surface	yes	10	98.3	7
Eukoenenia sp. 3***	Arma Cornarea	COR1	04/01/2018	alive	water surface	yes	–	–	–
	Arma Cornarea	COR1	04/01/2018	alive	water surface	yes	–	–	–
	Arma Cornarea	COR1	20/04/2019	alive	water surface	yes	11.7	94.5	7

**Eukoenenia* sp.1 – Balestra V. leg., sample damaged during preparation (E. Christian in litt.)

***Eukoenenia* sp.2 – Lana E. & Balestra V. leg., 1 male with a combination of characters that does not conform to any described species of NW Italy: 4 blades in the lat. organ; elevated number of a-setae on sternites IV-VI; tergites IV-VI with s-5t-s. ,1 juvenile female, indet. (E. Christian in litt.)

****Eukoenenia* sp.3 – Balestra V. & Marovino M. leg., 1 juvenile female, not determined to species level: Eukoenenia of the group spelaea (E. Christian in litt.)

Environmental/ecological data

Temperature (T °C) and relative humidity (RH%) data related to the presence of 43 living individuals in eight different caves for different species have been recorded (Table 2, Fig. 3A, B). *Eukoenenia* species in Western Italian Alps resulted to be present in a temperature range between 9.0 and 13.7 °C and in a range of RH% between 81 and 100%.

A negative correlation between temperature and relative humidity is observed for the presence of *Eukoenenia strinatii* (r: -0.9285) (Fig.3B). For this species, observation sites at low temperature have a higher RH% compared to the sites at higher temperature. Correlation between the two parameters was not calculated for the other species due to the paucity of data despite a potential trend, shown in Fig. 3A.

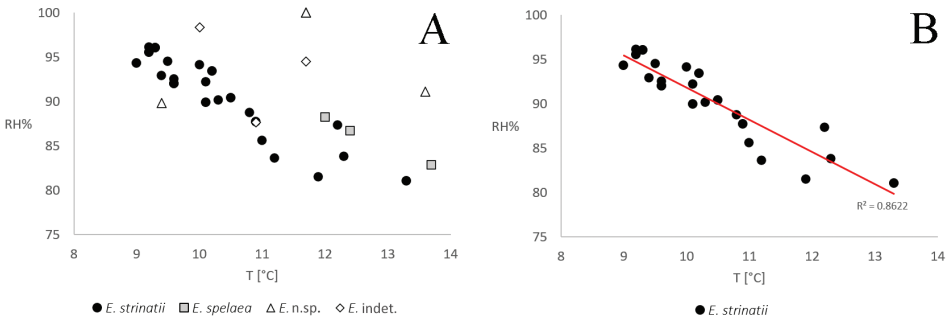

Figure 3. Relation between temperature and relative humidity in the micro-habitat of genus *Eukoenenia* in the Western Italian Alps. **A** *Eukoenenia* species **B** *E. strinatii.*

The pH of the water pools where 52 specimens were observed was close to neutral (pH 7). This result was obtained from 32 sampling sites, of which13 measurements were repetitions from the same site performed in different seasons confirming no seasonal variation (Table 2). The only exception was recorded from the Buranco di Bardineto cave, where the pH was nearly 6 in a single pool which was particularly rich in organic substance.

A total of 143 *Eukoenenia* individuals were observed on different microhabitat with the majority of them from water surfaces: one on wet wood, one under a stone, three near water, ten on the cave ground and 128 on the surface of pools that had calm water or a weak current (Table 2, Fig. 4, 5), especially in the rimstone dams, also called gours (Fig. 5A) – a particular type of speleothems (cave formation) in the form of a stone dam (Hill and Forti 1997). Moreover 16 dead individuals were observed but not

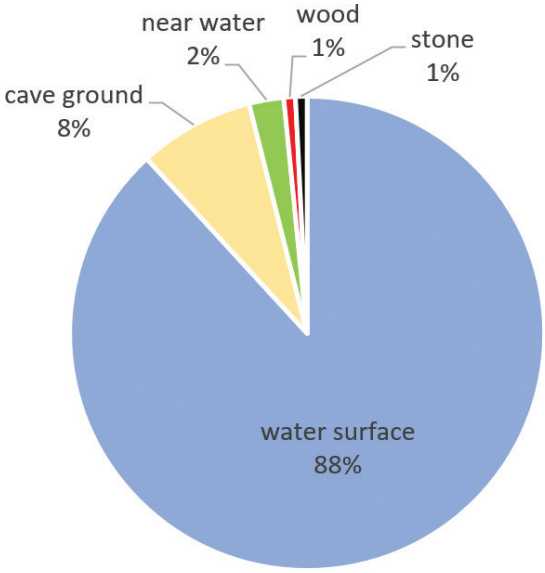

Figure 4. Percentage of specimens found on different microhabitats.

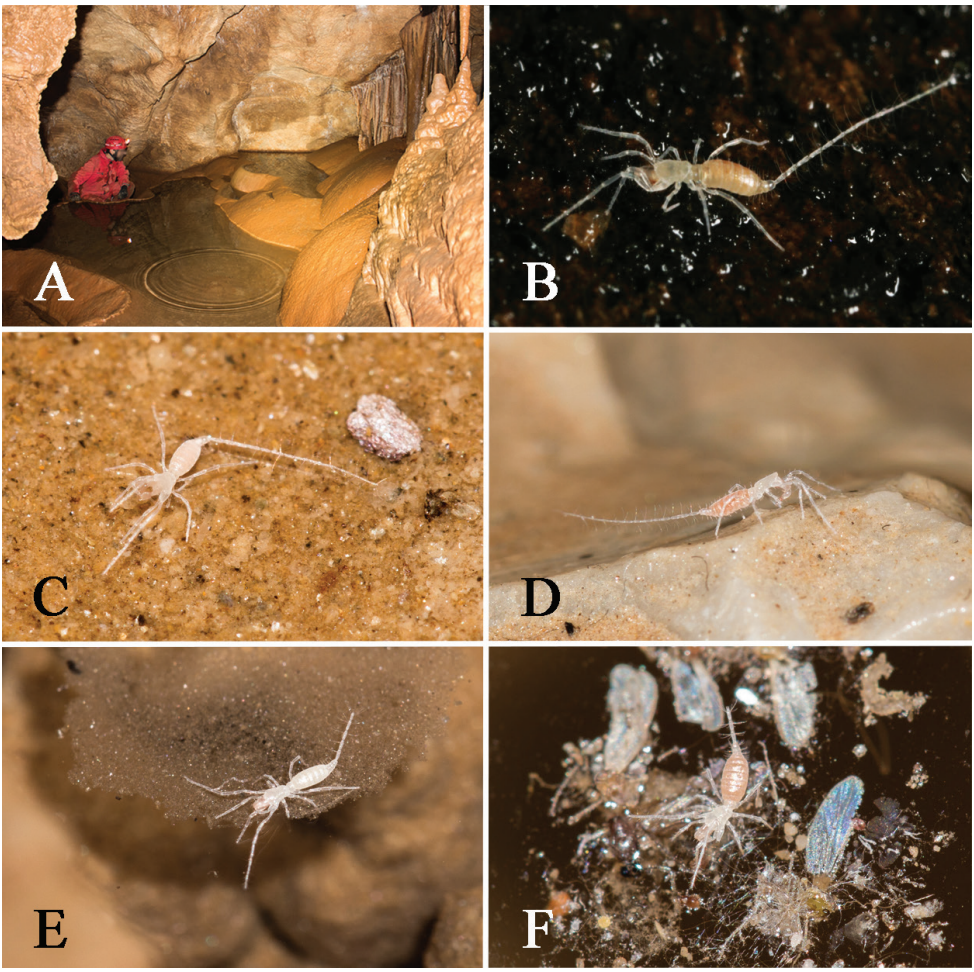

Figure 5. Habitat monitored and different microhabitat where *Eukoenenia* individuals were observed. **A** Rimstone dams (gours) in Buranco di Bardineto cave **B** *E. strinatii* on wood in Bossea cave **C** *E. strinatii* near water in Bossea cave **D** *E. strinatii* on Bossea cave ground **E** *E. strinatii* on raft of crystalline materials in Bossea cave **F** *E. strinatii* on raft of crystalline materials, fungal hyphae and organic remains in Bossea cave. (photos **A, C, D, E, F** by V. Balestra, **B** by E. Lana).

considered for the ecological interpretation of the taxon and it has not been possible to define the way in which they reach the place they were observed.

A significant association between *Eukoenenia* individuals and environments rich in organic matter was observed (Yates' Chi square: 62.41, $p < 0.000$), in fact, in the rimstone dams, where 112 living *Eukoenenia* individuals were sampled, trophic resources were abundant. Cave rafts, that are mainly calcite crystals, are common on the surface of quiescent waterbody such as the rimstone dams (Hill and Forti 1997) (Fig. 5E) and they often "trap" dead animals, fungal hyphae and organic remains (Fig. 5F).

Eukoenenia individuals were observed in different sampling areas ranging from a few meters to more than 500 m from the entrance. However, due to the sampling method and the structure of the investigated caves, no conclusion can be drawn about the effect of the distance from the entrance to the taxon distribution.

In addition, it is worth mentioning that *E. strinatii* was also observed in 3 sampling areas in the touristic Bossea cave where light, even if not direct or continual, was present.

Observation on trophic role of the taxon and feeding behaviour

As previously mentioned, an association between the presence of living specimens and the presence of organic matter on the water surface was demonstrated in the sampling area. Individuals of *Eukoenenia* were also observed feeding on different species of dead springtails. In particular, in June 2016, an *E. strinatii* individual was observed and photographed for the first time feeding on dead springtail (Balestra et al. 2019; Lana et al. 2016) (Fig. 6). The observed specimen (observation made through Canon macro lens, 65 and 100mm, f/2.8) moved on the surface of the water in an unusual way, using the three pairs of hind legs and the palps on the liquid, keeping up the legs of the first pair stretched forward and the flagellum in a vertical position (Fig. 6A–D). The arachnid approached a dead springtail, *Pseudosinella alpina* Gisin, 1950, and began to suck its internal liquids having damaged the integument of the corpse in the abdomen with the chelicerae; a drop of exudate came out in which *Eukoenenia* immersed the chelicerae and approached the mouthparts (Fig. 6E–G). The drop of exudate was consumed in about 15 minutes and then the specimen moved away from its meal with a particular walking: with short steps, with the paws of the second and third pair and the palps aligned, the paws of the first pair raise and face forward and the abdomen is raised with the flagellum pointing upwards (Fig. 6H). In addition, in June 2018, in Rio Borgosozzo cave, a specimen of *E. strinatii* that carried a dead springtail, holding it with chelicerae, was photographed (Fig. 7). While moving, it held the same position of the *E. strinatii* as previously described from the Bossea cave after its meal.

In contrast with the feeding activity of *Eukoenenia* on dead springtail specimens and despite the observed proximity between specimens of *Eukoenenia* and living and floating entomobrid Collembola apart from rare phenomena of palpation with the paws of the first pair, no attacks by the palpigrades on the living springtails were observed, recorded and documented.

On the contrary, in one case we observed and documented a potential response of a springtail against *Eukoenenia* (Fig. 8). After several palpations by the palpigrade on two springtails (Fig. 8A, B), *E. strinatii* tried to climb on one of the springtails (Fig. 8C), which in reaction, potentially bit the arachnid in the lower part of the body. The springtail moved to another part of the gour and for a few moments *E. strinatii* remained lying on its side, touching the mesosoma with its chelicerae (Fig. 8D). Later the arachnid got up and started to walk again on the surface of water.

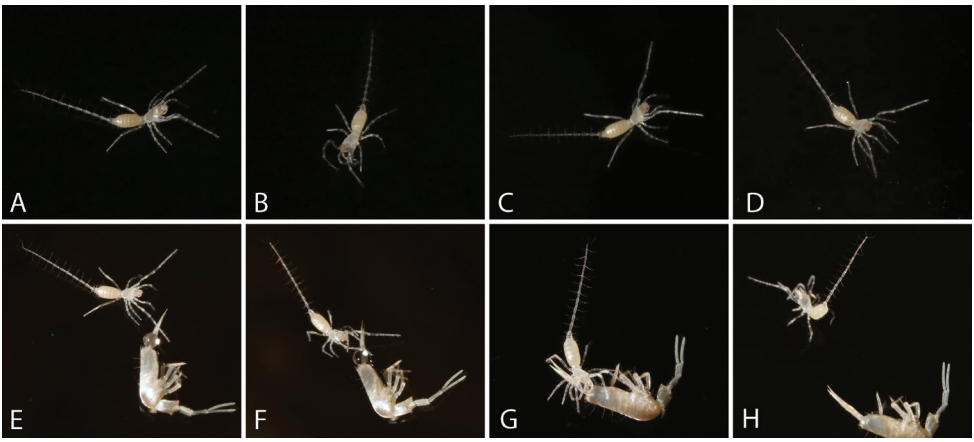

Figure 6. Approach and meal of *Eukoenenia strinatii* on *Pseudosinella alpina* (photos by E. Lana).

Figure 7. Meal of *Eukoenenia strinatii* on dead springtail (photo by V. Balestra).

Figure 8. Approach and bite of *Eukoenenia strinatii* and two living Collembola (photos by V. Balestra).

Discussions

In the last year, due to the high sampling effort, the level of knowledge about the distribution of the species of the genus *Eukoenenia* in Western Italian Alps has been exponentially increased: 1) more than 100 individuals were observed and documented in the last four years; 2) before 2016 palpigrade specimens were observed only in seven caves, whereas today *Eukoenenia* species are reported from 17 caves.

Therefore, it has been possible to increase the number of sites where *Eukoenenia* species is present, both in term of caves and in term of areas within caves. For example, *Eukoenenia strinatii* was considered endemic in the Bossea cave until 2016, now this species is reported from other four caves and from different areas within the Bossea cave (from seven in 2016 to 10 in 2021) (Balestra et al. 2019; Condé 1977; Morisi 1992).

The data reported here highlights the fact that palpigrades in Western Italian Alps seem to be not so rare: probably the paucity of data on these organisms was due to the difficult access to some habitats (Ficetola et al. 2019; Howarth 1983; Mammola 2019) and to the lack of knowledge of the environmental parameters that define the preferred habitat for this taxon. Moreover, in (Mammola et al. 2021b) a significant species-people correlation effect (number of species/number of researchers studying palpigrades) has been described, showing that the concentration of palpigrade records in some regions of the globe can be influenced by the presence of researchers interested in this group.

As for other species of invertebrates present in the Bossea cave, palpigrades do not seem to be particularly and negatively influenced by tourism. Despite their troglomorphism they tolerate the presence of lights, even if not direct, and probably only for brief periods. The presence of palpigrades in touristic cave was also observed in the Brazilian Maquiné cave, where *E. maquinensis* Souza & Ferreira 2010 is reported (Ferreira and Souza 2012).

Temperature and relative humidity values recorded in this study are typical of Alpine caves, however, the sampling data revealed the absence of *Eukoenenia* in cold caves (T < 8 °C). The most favorable caves for *Eukoenenia* genus seem to be those with mild temperatures and very high relative humidity, or higher temperatures and lower relative humidity. The parameters monitored in Maquiné cave, Brazil, showed higher temperature (23.6 °C to 24.5 °C) but similar RH% (89 to 95%) for *E. maquinensis* (Ferreira and Souza 2012).

Palpigradi living in the hypogean environment of the Western Italian Alps have usually been observed on the surface of pools of rimstone dams. They have rarely been observed on wet wood, near water, on wet stones or on the cave ground. This does not mean that the favored environment of these small arachnids is the water surface of the underground pools, on which they probably can easily float thanks to the surface tension of the liquid or with rafts, but that this possibly reflect higher detectability in this cave habitat. Their main habitats can be those interstitials (Howarth 1983; Mammola 2019) and probably they could be transported in underground pools by water flow during rainy periods, getting trapped on the rafts, or they could voluntarily go into this environment in search of food. In fact, relying on different observations on individuals on the water surface, the presence of trophic resources in 90% of cases

makes it reasonable to think that food availability is the main factor influencing *Eukoenenia* specimens to venture on the rafts and in the gours.

In more than 100 direct observations in caves it was never possible to document an attack of palpigrades on a prey. An approach on dead springtails sucking their internal liquids have been observed. If the ingestion of liquid food is their feeding system, as observed in other arachnids, it could be explained why no solid remains were found in the digestive tract of the palpigrades (Condé 1984; Millot 1942). A direct attack on collembola in caves was documented (Lukić 2012) and a specimen of *E. strinatii* that carried a dead springtail, keeping it with chelicerae, was photographed in this work. Moreover, a recent study on gut content of *E. spelaea* has shown the presence of spiders, beetles, mites, springtails, and flies DNA (Parimuchová et al. 2021), supporting carnivory in palpigrades. Wheeler (1900) suggested that palpigrades probably feed on eggs or juvenile stages of bigger arthropods invertebrates, however, our observations on scavenging activity could explain the presence of genetic material of large arthropods in the intestinal tube of palpigrades.

The combination of our observations and the data from other authors support the idea that palpigrades are predator and scavengers depending on the food resources availability, due to the fact that caves are extreme environments with limited trophic resources. Cyanobacteria and Fungi could be an alternative food source or, probably, an accidental consumption due to the ingestion of contaminated prey, as suggested by Parimuchová et al. (2021).

Conclusion

In conclusion the results of this study highlights that:

i. The hypogean palpigrades in the Western Italian Alps seems to be anything but rare and the paucity of records of this taxon is related to sampling bias. A correct knowledge of the preferred habitat and the environmental conditions where they live allows the observation of a relevant number of specimens.

ii. Hypogean palpigrades were observed in distinct regions inside caves, located at different altitudes, including records in areas with artificial lighting for tourist access. The presence of these animals is mainly influenced by temperature, relative humidity, trophic resources and the presence of two speleothems: rimstone dams and rafts. The most favorable cave habitats for *Eukoenenia* genus seem to be those with mild temperatures and very high relative humidity, or higher temperatures and lower relative humidity whereas the taxon was not recorded from cold caves.

iii. Palpigrades are predators and scavengers depending on the food resources availability due to the fact that caves are extreme environment with limited trophic resources.

Caves are special environments that host unique creatures in a world still to be explored and documented. In this study, it was possible to provide a first knowledge on the environmental parameters that can influence the distribution of hypogean palpigrades in Western Italian Alps. Moreover, the feeding behaviour in palpigrades

is directly observed and photographed for the first time. Direct observation and photographs can be very useful to elucidate biological aspects of fragile groups that are difficult to keep under laboratory conditions, such as palpigrades. This research can be considered the starting point for future and more detailed studies on this curious genus and other apparently rare hypogean taxa in Italian caves.

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RESEARCH ARTICLE

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Transitional and small aquatic cave habitats diversification based on protist assemblages in the Veternica cave (Medvednica Mt., Croatia)

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Abstract

Protists in caves are scarcely researched. Most cave studies address the diversity of protists, but very little is known about their habitats and spatio-temporal dynamics. The aim of this study was to investigate the diversity and abundance of protists in the Veternica cave in the Medvednica mountain in Croatia on hygropetric and sinter and clay pools during six months. During this study, 47 protists taxa were distinguished belonging to the groups of heterotrophic flagellates, ciliates, heliozoans, stramenopiles and testate and naked amoebae. The highest taxon richness was found at the sinter pool site richest in bats guano (36 taxa). Most diverse were testate amoebae and ciliates. The number of taxa and their abundance differed significantly between the sampling sites. The prospected habitats supported different protist assemblages, proved by ordination analysis. The most distinctive habitat was the hygropetric. Habitat heterogeneity could be attributed to the presence of inorganic and organic sediments at the sites and habitat microhydrology (lotic or lentic system). Kendall's concordance coefficient showed a good synchronicity between the habitats in the Veternica cave, based on taxon richness and abundance of protists indicating similar seasonal trends. Seasonality in the studied habitats is attributed to the hydro-meteorological conditions in the Veternica cave drainage area. This study is one of the few studies of spatio-temporal diversity and abundance of protists in caves. Despite the similar appearance of small transitional and aquatic habitats in caves, an example of this study showed specific habitat diversification.

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Keywords
Bat's guano, cave protists, clay pools, habitat heterogeneity, heliozoan, hygropetric, seasonality in cave, sinter pools, spatio-temporal dynamics, testate amoebae

Introduction

Freshwater karst caves are formed in soluble rock such as limestone or dolomites by dissolution processes induced by rainwater that is slightly acidic by dissolved CO_2 from the atmosphere and also biogenically produced within soil layers (Ford and Williams 2007). In opposite to cave entrances, the deeper parts of the caves have relatively constant microclimatic conditions (temperature, high humidity) and absolute absence of sunlight. The subterranean karst habitats are extending, not only within caves, but also in other parts of the bedrock where dissolution has created voids large enough to be inhabited by living organisms (Culver and Pipan 2009). In other words, caves are just small windows into much larger habitats: the vadose and phreatic zone of the karstified bedrock.

The majority of current cave research was focused on animals that are often highly endemic and exhibit morphological, behavioural and physiological adaptations to their environment (Culver and Pipan 2009). Other organisms in these ecosystems, such as fungi and various microorganisms have been neglected and very little data is known about their biodiversity and ecology (Gottstein Matočec et al. 2002; Moldovan et al. 2018).

Nevertheless, many researchers have made contributions to our knowledge of cave protists, even though these are mostly based on on-off sampling. To date, several hundred species of cave protists from all continents have been discovered. In their comprehensive work, Gittleson and Hoover (1969, 1970) reviewed the literature on protists in caves around the world, and reported more than 400 species. Researchers from Italy contributed predominantly to the knowledge of ciliates in caves (Coppellotti and Guidolin 1999; Coppellotti Krupa and Guidolin 2003; Luca et al. 2005) and studies of naked (Bastian et al. 2009; Walochnik and Mulec 2009; Mulec et al. 2016) and testate amoebae (e.g. Chibisova 1967; Golemansky and Bonnet 1994; Mazei et al. 2012; Baković et al. 2019) were also done.

Most protists species found in caves have been previously registered in non-cave habitats, and many of them were allocated as euribiotic species (Golemansky and Bonnet 1994). Although few protists species or forms have been described from caves (Chibisova 1967; Walochnik and Mulec 2009), some of them have been found later in surface habitats or have been found as synonyms with previously described species.

The ecology of cave protists has very scarcely been investigated. The main gaps in studies on the subterranean protist ecology is the lack of quantitative methods, as well as precise and thus comparable habitat characterisation (e.g. Vandel 1965; Gittleson and Hoover 1969). Comparing data from particular habitats is also complicated by inconsistency in terminology and by often inadequate species descriptions that make correct identification impossible (Lahr et al. 2012; Kosakyan et al. 2016). Also, it is not always clear whether samples were taken within the cave entrance, where light was also present and affecting the communities, or in a truly subterranean habitat. The diversity

of protists was studied in mostly aquatic habitats like subterranean streams, lakes and various smaller water pools that formed inside the caves (Vandel 1965; Gittleson and Hoover 1969, 1970). Some data refer to cave sediments and transitional habitats characterized by water layers present on solid surfaces, biofilms and fungal mycelia biomass (Golemansky and Bonnet 1994; Coppellotti and Guidolin 1999; Coppellotti Krupa and Guidolin 2003; Bastian et al. 2009; Garcia-Sanchez et al. 2013). Epibiotic protists were registered on cave-dwelling animals (Matjašič 1962; Dovgal and Vargovitsh 2010).

This research represents the continuation of the study of protists in the Veternica cave in the Medvednica mountain in Croatia. The impact of hydro-meteorological conditions on the protist assemblages in this cave was discussed by Kajtezović and Rubinić (2013). Findings of some protists within the lampenflora in the Veternica cave were mentioned by Baković (2016). Baković et al. (2019) described a new species of testate amoebae, *Centropyxis bipilata*, from caves of the Dinaric Arc, which was also present in the Veternica cave.

The aim of this study was to investigate the spatio-temporal taxon richness and abundance of protists present in the Veternica cave. We also compared various habitats in order to test how habitat heterogeneity within this cave affects protist assemblages. This research is, to our knowledge, one of the few studies of spatio-temporal changes in taxon richness and abundance of protist taxa in freshwater karst caves.

Methods

Study site

The Veternica cave is a complex speleological object located in the southwestern part of the Medvednica mountain (45°50'27.22"N, 15°52'24.75"E) at an altitude of 330 m above sea level (Fig. 1). The full length of the horizontal cave channels is 7,128 m (Fig. 2). It was dominantly formed between the Triassic dolomite and Miocene limestone (Lacković et al. 2011). The first 380 m of the cave is open for organized tourist visits from March to November. In winter, this cave is a nationally important hibernaculum for bats with over 1000 individuals, and in summer there is one maternity colony present near the cave entrance (Petra Žvorc, pers. comm.). In this cave there is air circulation during the summer months (blowing out of the cave) and winter months (outside air entering the cave).

The research was conducted from October – December 2011 and February – April 2012 at six cave sites with three habitat types: sinter pools (site SP-1, SP-2), clay pools (site CP-1, CP-2) and hygropetric (site HP-1, HP-2). Sampling at sites SP-1 and CP-1 was not performed in October 2011 due to technical difficulties. All sampling sites were out of reach of sunlight. During the research, pre-hibernation and hibernation colonies of bats (mostly *Rhinolophus* spp.) were present.

Sinter pools (SP) in this investigation represented small cascade pools on speleothems filled with water. They were created by gradual precipitation and dissolution of calcium carbonate from seeping water. At this specific location, cascades of sinter pools were of very small depth, ranging from a few millimetres to several centimetres. Slowly dripping water from the speleothems above this habitat contributes to the constant presence of wa-

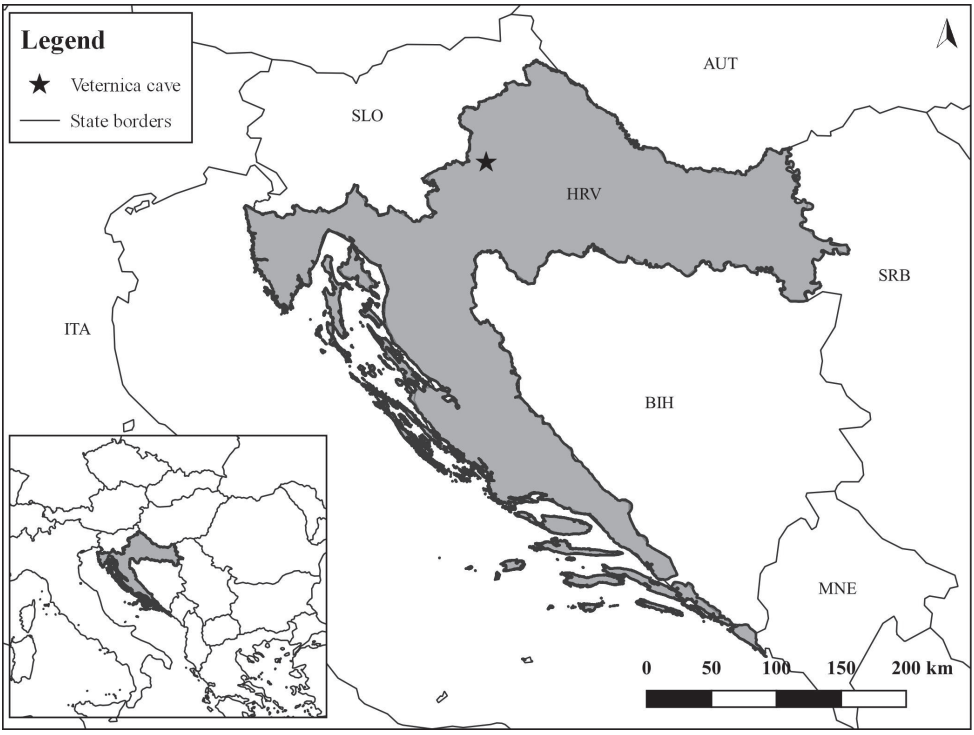

Figure 1. Location of the Veternica cave.

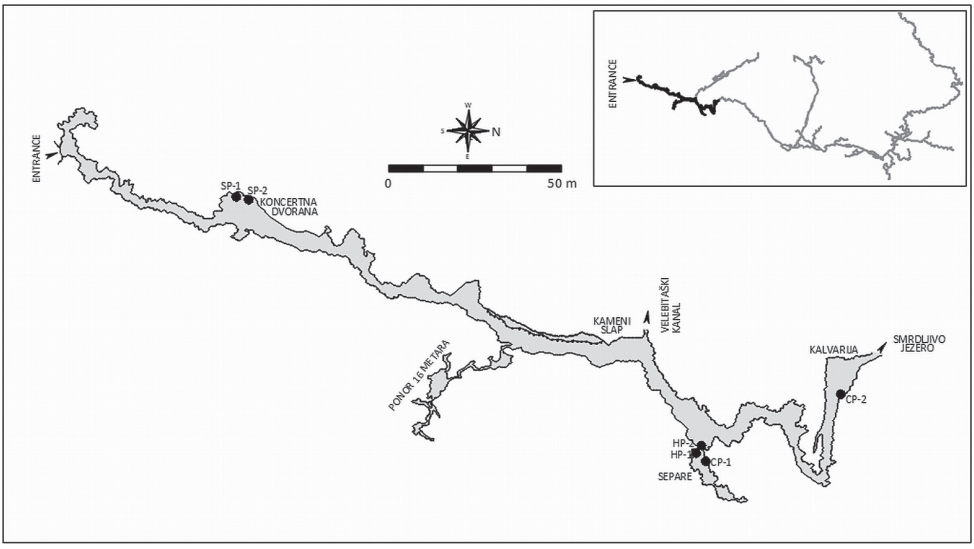

Figure 2. Map of the Veternica cave [adapted from Čapelak (1979)] with marked sampling sites (CP-clay pools; SP-sinter pools and HP-hygropetric).

ter. Samples were taken at Koncertna dvorana, located in the main cave channel, at a distance of 90 m from the cave entrance; the distance between SP-1 and SP-2 is about 1.5 m.

Clay pools (CP) are small pools in cave clay deposits filled with water. They are created by the constant dripping of water from the speleothems onto clay deposits. The depth of these pools varies from a few millimetres to several centimetres. Site CP-1 was located in a small side channel named Separe (about 1.8 m from stations HP-1 and HP-2), the distance to the cave entrance is about 250 m. Site CP-2 was located in a hall named Kalvarija, at a distance of about 380 m from the cave entrance. The distance between site CP-1 and CP-2 was about 100 m.

Hygropetric (HP) is a habitat type characterized by a thin layer of water (~1 mm) constantly seeping onto the surface of speleothems. Location in the cave: a small side channel named Separe, distance to the cave entrance is about 250 m; distance between sites HP-1 and HP-2 is 1.2 m.

Sampling, measurements and identification

Cave air temperature and cave relative air humidity were measured using the Kestrel 3000 instrument.

Plastic containers were filled with 40 ml of tap water. Water and bottom sediment from sinter and clay pools were transferred into the prepared containers using graduated plastic pipettes. Additionally, solid surfaces of sinter pools were rubbed with plastic brushes and the collected sediment was afterwards transported into the plastic containers by repeated "washing" the brush in the collected water in the container. Samples from the cave hygropetric were collected by rubbing its surface with plastic brushes and repeated "washing" the brush in the water in the container.

The collected samples contained water, mineral particles and bats guano (if present in the habitat). Samples were maintained at temperatures of 4–10 °C and prospected within 48 hours after sampling. After decantation, a mixture of sediment and water was taken and a triplet of 0.2 ml (total 0.6 ml) samples were prospected and data on diversity of protist taxa were noted, together with the taxon richness of the accompanying meiofauna. The number of individuals of each protist taxa in prospected volume (total of 0.6 ml) was counted. The abundance of protist taxa in 1 ml was estimated based on the data of the abundance in 0.6 ml of prospected volume. Diversity and abundance were estimated using a Carl Zeiss Primostar light microscope. The presence of guano in the collected sample was estimated by using insects leftovers as indicators (exoskeleton fragments, butterfly wings scales) and their aggregations on the microscopy slide (present in fresh guano deposits rich also in non-chitin material). It was rated according to the following scale: 0 – absent (no traces of insects leftovers), 1 – low quantity (up to ten insects leftovers in the sample, leftovers are not aggregated), 2 – medium quantity (eleven or more insects leftovers present, leftovers are not aggregated), 3 – high quantity (aggregation of insects guano is present regardless of number of insects leftovers).

The samples were scanned for protists using a Nikon Diaphot inverted light microscope and a Carl Zeiss Primostar. Selected cells were examined in detail with the

Primostar in higher magnifications or with an Olympus BX51 light microscope with differential interference contrast (DIC) and Phase Contrast optics.

Additional samples were collected in July 2020 to confirm the identification of heliozoans from the sinter pools (SP). Drops of this material were pipetted onto light microscope slides and covered with a slip. These slides were kept in a humidity chamber for several days and observed for heliozoans and naked amoebae. When heliozoans had established their populations after several days, the slip was gently removed and the slide was left to dry. This air-dried material, containing plate and spine-scales of heliozoans, was photographed with FEM-REM Zeiss Neon by Steffen Clauß in Chemnitz (Germany) to facilitate their identification.

Specimens were identified by genus and, if possible, by species level, mainly based on the following references: ciliates: Kahl 1930; Foissner et al. 1991, 1992, 1994, 1995; Foissner and Berger 1996; naked amoebae and heliozoa: Page and Siemensma 1991; testate amoebae: Ogden and Hedley 1980; Mazei and Tsyganov 2006; Tsyganov et al. 2016.

Data analysis

Data about the taxon richness and abundance between the sampling sites were analysed using descriptive statistics in Statistica software and MS Office Excel.

Differences in the number of taxa and the abundance between the sites were analysed using Friedman's ANOVA, a nonparametric alternative of one-way repeated measures ANOVA. The analyses were done in Statistica 13 (TIBCO Software Inc.). Similarities between protist assemblages were tested using the Bray-Curtis similarity, which was used for nMDS analysis. The latter analysis was done in PRIMER6 (PRIMER-e Ltd).

Results

The largest variability of the microclimate in the Veternica Cave was recorded in Koncertna dvorana – the air temperature ranged from 4.1 °C (Feb 2012) to 10.5 °C (Oct 2011), while the relative air humidity ranged from 70.7% (Mar 2012) to 91.5% (April 2012). The air temperature in Separe ranged from 8.1 °C (Dec 2011) to 10.7 °C (Oct 2011), while the relative air humidity ranged from 77.7% (Feb 2012) to 81.6% (Nov 2011). The air temperature in Kalvarija ranged from 7.5 °C (Feb 2012) to 11.2 °C (Oct 2011), while the relative air humidity ranged from 82.2% (Oct 2011) to 90.8% (April 2012).

A total of 47 taxa of protists belonging to heterotrophic flagellates, ciliates, heliozoans (Fig. 3) and testate (Fig. 4) and naked amoebae were recorded (Table 1). Protists were not registered in the samples from HP-1 on 2/2012 and HP-2 on 12/2011. Taxon richness was highest within ciliates and testate amoebae (both 16 taxa). Less diverse groups were naked amoebae (6), heterotrophic flagellates (6), heliozoans (2) and stramenopiles (1).

After six months of research, the sites with the highest taxa number were SP-2 (36 taxa), SP-1 and CP-1 (both 20 taxa). A somewhat lower taxon richness was recorded at site CP-2 (17 taxa).

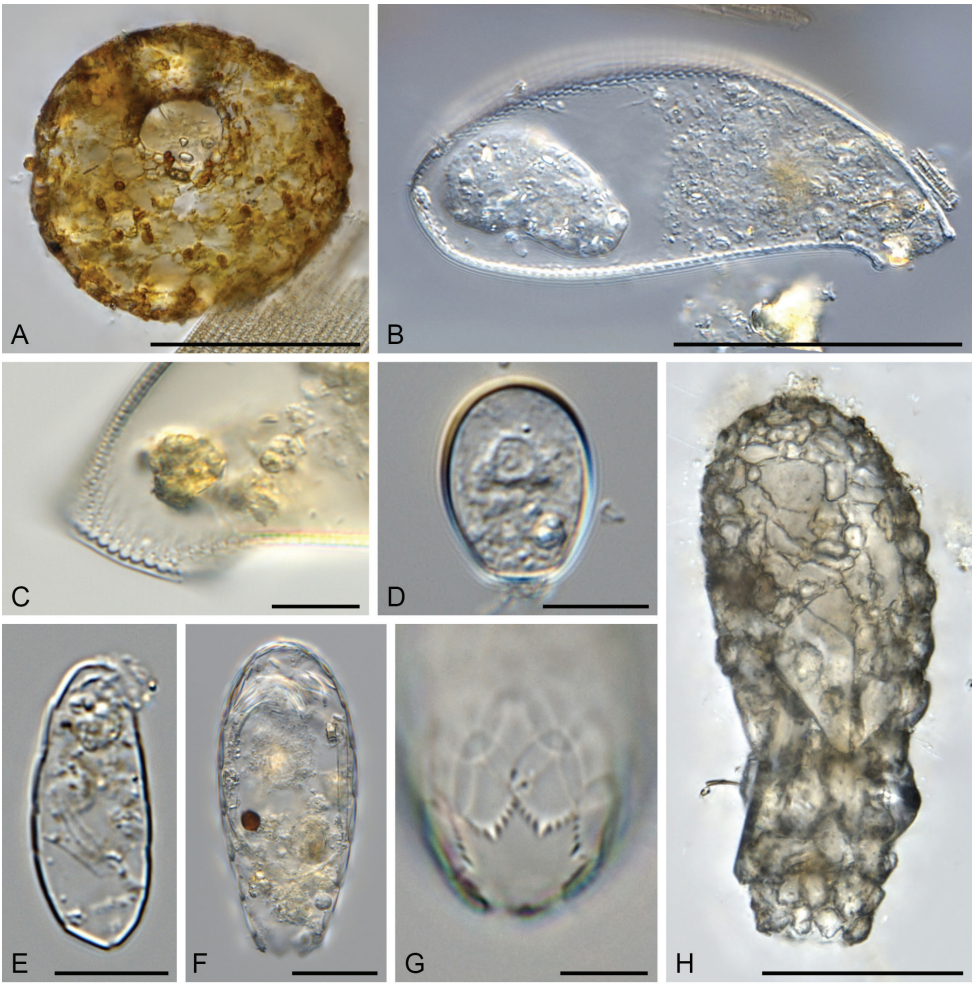

Figure 3. A *Centropyxis bipilata*; **B, C** *Cyphoderia ampulla* **B** test **C** collar **C** *Cryptodifflugia oviformis* **E** *Trinema lineare* **F, G** *Euglypha tuberculata* **F** test **G** aperture **H** *Difflugia oblonga*. Scale bars: 50 µm (**A, B, H**); 20 µm (**F**); 10 µm (**C–E, G**); light microscopy.

Occasionally, resting stages (cysts) morphologically typical of amoeboid protists were found at sites SP-1, SP-2 and CP-1. Some of them were present in testate amoebae shells.

Meiofauna was occasionally registered in all habitats: SP (Rotifera, Nematoda, Turbellaria, Copepoda, Oligochaeta, Hydrachnidia, insect larvae), CP (Rotifera, Nematoda, Hydrachnidia, Oligochaeta, insect larvae) and HP (Nematoda, Hydrachnidia, Copepoda).

Taxon richness and abundance of protists were higher in habitats SP and CP, compared to HP (Fig. 5; Table 2). The standard deviation of the SP and CP habitats was also greater than of the hygropetric habitats. The number of taxa differed significantly between sites (Friedman's ANOVA, $\chi(n = 5, d.f. = 5) = 17.53$, $p < .01$), with Kend-

Table 1. Protist taxon richness on research sites.

Taxa	Research sites					
	SP-1	SP-2	CP-1	CP-2	HP-1	HP-2
Testate amoebae						
Arcella artocrea Leidy, 1876	-	+	-	-	-	-
Arcella rotundata Playfair, 1918	-	-	-	-	-	+
Centropyxis aerophila Deflandre, 1929	+	-	-	-	+	+
Centropyxis bipilata Baković, Siemensma, Baković, Rubinić, 2019	-	+	-	-	+	-
Centropyxis laevigata Penard, 1890	-	+	-	-	-	-
Cryptodifflugia oviformis Penard, 1902	+	+	+	-	-	-
Cyclopyxis sp.	+	-	-	-	+	+
Cyphoderia ampulla Ehrenberg, 1840	-	+	-	+	-	-
Difflugia oblonga Ehrenberg, 1838	-	+	-	-	-	-
Difflugia sp.	-	+	-	-	-	-
Euglypha laevis (Ehrenberg, 1832) Perty, 1849	+	+	+	+	+	+
Euglypha rotunda Wailes, 1915	-	+	-	-	-	-
Euglypha sp.	+	-	-	-	-	+
Euglypha tuberculata Dujardin, 1841	-	+	-	+	-	-
Tracheleuglypha dentata Deflandre, 1928	-	+	-	+	-	-
Trinema lineare Penard, 1890	+	+	+	+	+	-
Heliozoa						
Acanthocystis myriospina Penard, 1890, emend. Dürrschmidt, 1985*	-	+	-	-	-	-
Raphidocystis marginata (Siemensma, 1981) Zlatogursky, 2018*	+	+	+	+	-	-
Ciliophora						
Ciliophora 1	-	+	-	-	-	-
Ciliophora 2	-	+	-	-	-	-
Ciliophora 3	-	-	+	-	-	-
Cinetochilum margaritaceum Perty, 1849	+	+	+	+	-	-
Colpoda sp.	-	-	+	-	-	-
Colpoda steini Maupas, 1883	-	+	+	+	-	-
Cyclidium glaucoma O.F.M., 1786	+	+	+	-	-	-
Cyrtophoryda	-	+	-	-	-	-
Glaucoma sp.	+	+	+	+	-	-
Hymenostomata 1	-	+	+	+	-	-
Hymenostomata 2	-	-	-	-	+	+
Litonotus lamella Schewiakoff, 1896	-	+	-	-	-	-
Nassulida 1	+	+	+	+	-	+
Nassulida 2	-	-	-	-	-	+
Pleuronema sp.	+	+	+	+	-	-
Sphatidium sp.	-	-	+	-	-	-
Stramenopiles						
Actinophrys sol Ehrenberg, 1830	+	+	+	-	-	-
Heterotrophic flagellates						
Nanoflagellata (five taxa)	+	+	+	+	+	+
Peranema trichophorum (Ehrenberg 1838) Stein 1878	+	+	-	-	-	-
Naked amoebae						
Amoebozoa 1	-	+	-	-	-	-
Amoebozoa 2	+	-	-	-	-	-
Korotnevella sp.	-	+	-	-	-	-
Mayorella sp.	-	+	-	-	-	-
Rhizamoeba sp.	+	-	+	-	-	-
Vahlkampfia sp.	-	+	-	-	-	-

*In addition, specimens of the heliozoan *Raineriophrys erinaceoides* (Petersen & Hansen, 1960) Mikrjukov, 2001 were found in the 2020 samples.

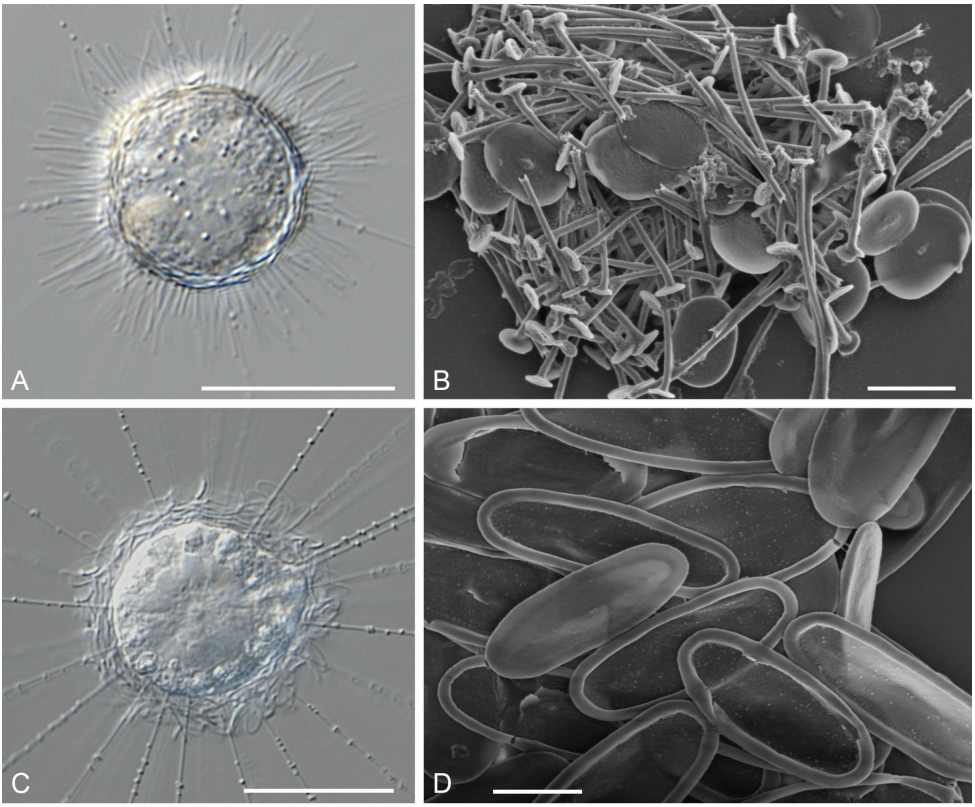

Figure 4. A, B *Raphidocystis marginata* **A** LM micrograph of a living cell **B** REM micrograph of plate-scales and spine-scales **C, D** *Acanthocystis myriospina* **C** LM micrograph of a living cell **D** REM micrograph of plate-scales. Scale bars: 20 µm (**A, C**); 2 µm (**B, D**); LM, light microscopy; REM, raster electron microscopy.

all's concordance coefficients (τ) of .70 indicating good synchronicity between sites. There was also significant difference in protist abundance between the sites (Friedman's ANOVA, $\chi(n = 5, d.f. = 5) = 17.34$, $p < .01$). As with the number of taxa, there was a good synchronicity between all habitats ($\tau = .69$).

The composition of protist assemblages (Table 1) shows that ciliates, testate amoebae and flagellates were present in all habitats researched. In habitats SP and CP, dominant taxa were ciliates, testate amoebae and flagellates, but with unclear dominance within the individual site. Naked amoebae, stramenopiles and heliozoans were not recorded in the hygropetric at all. When present, they were less dominant compared to other groups. Naked amoebae were absent in CP-2.

In habitat HP testate amoebae were the dominant group with 63.6% (HP-1) and 50% (HP-2) of taxa, followed by the flagellates and ciliates.

Non-metric MDS plot, based on Bray-Curtis similarity between protist assemblages, suggested possible differentiation within prospected habitats (Fig. 6). The most distinctive was the hygropetric habitat. The similarity was somewhat higher within sinter pools, while clay pools showed some lower distinction.

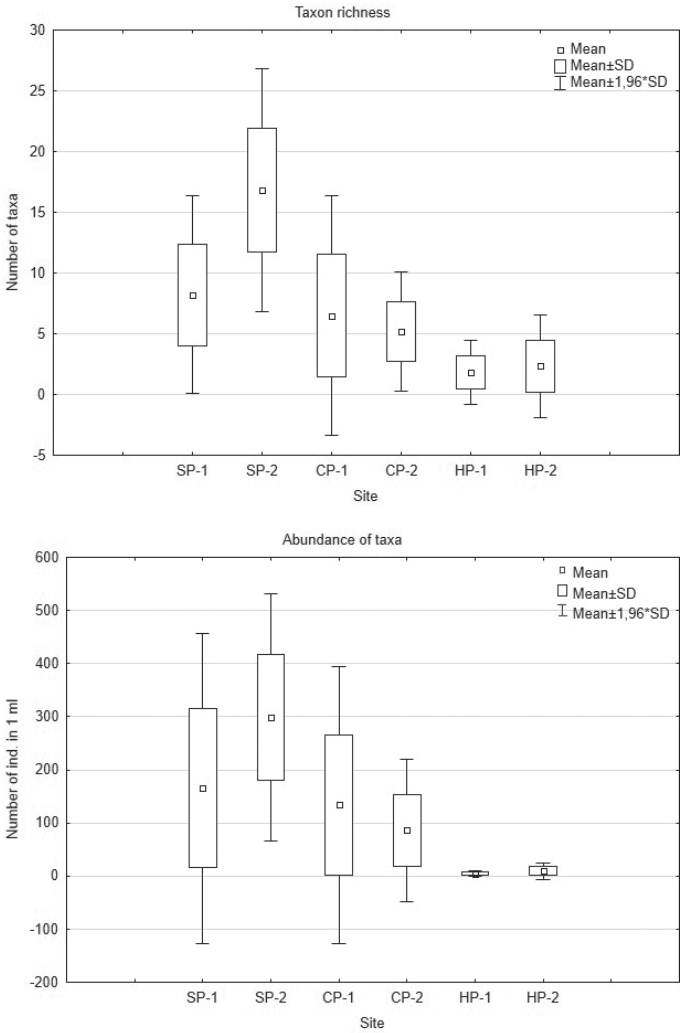

Figure 5. Taxon richness and abundance of protist taxa at the sites investigated.

Table 2. Abundance of protist taxa at the investigated sites.

Date	Protist taxa abundance (number of individuals in 1 ml) on research sites					
	SP-1	**SP-2**	**CP-1**	**CP-2**	**HP-1**	**HP-2**
October 2011	n/a	270.00	10.00	n/a	8.33	3.33
November 2011	91.67	220.00	256.67	3.33	5.00	18.33
December 2011	288.33	351.67	278.33	131.67	5.00	0
February 2012	75.00	191.67	1.67	80.00	0	20.00
March 2012	13.33	246.67	30.00	41.67	6.67	8.33
April 2012	358.33	513.33	226.67	173.33	3.33	6.67
Number of samples	5	6	6	5	6	6
Minimum	13.33	191.67	1.67	3.33	0	0
Maximum	358.33	513.33	278.33	173.33	8.33	20.00
Average	165.33	298.89	133.89	86.00	4.72	9.44
Median	91.67	258.33	128.33	80.00	5.00	7.50

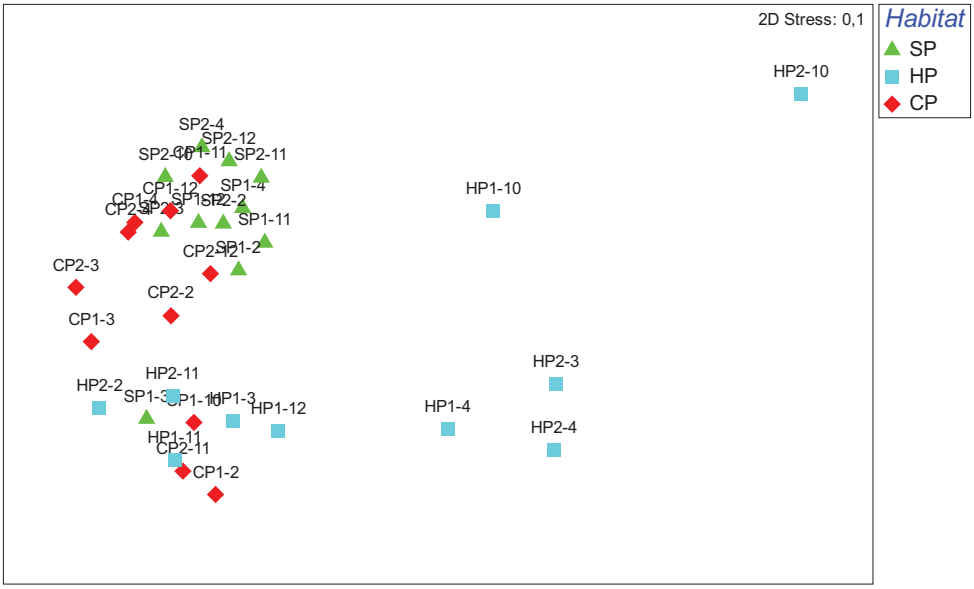

Figure 6. nMDS based on Bray Curtis similarity between samples.

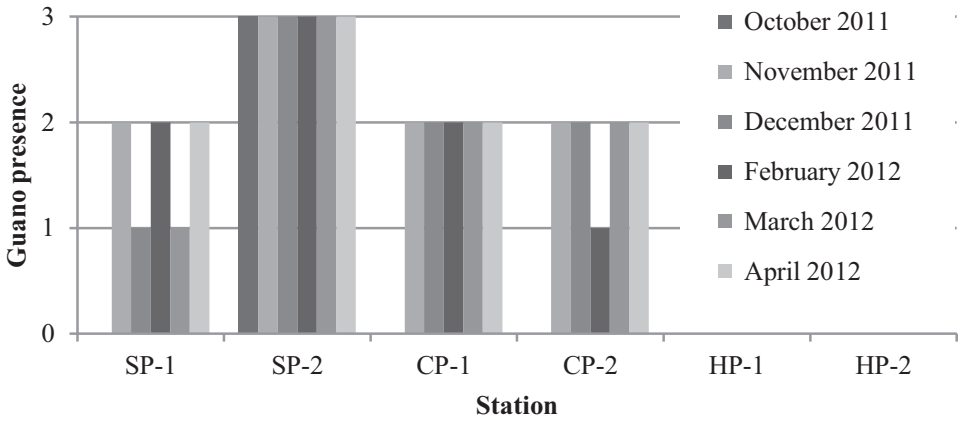

Figure 7. Presence of guano on researched habitats (0 – absent, 1 – low quantity, 2 – medium quantity, 3 – high quantity).

Guano was absent in the hygropetric, while in other habitats it was present in variable quantities (Fig. 7). Only in SP-2, guano was of high quantity during the whole research.

Discussion

In this research, 47 taxa of protists (Table 1) were distinguished in the Veternica cave in a short period of six months and in only six sites (3 habitats). This result implies that the

protist taxon richness could be higher compared to other animals inhabiting the caves. The six-month research of protists in the Mexican cave Cueva de Los Riscos (Sigala-Regalado et al. 2011) revealed only 13 taxa of protists. The authors did not report of testate amoebae, even though they are very diverse in subterranean habitats (Mazei et al. 2012). On the other hand, the taxon richness of testate amoebae recorded in the Veternica cave during this research showed that they were present in all habitats and stations. The research in caves of the Dinaric Arc (south-eastern Europe) showed that the biodiversity of testate amoebae varies depending on the individual cave and sampling efforts (Baković et al. 2019). Testate amoebae diversity in caves from Italy and Russia was also higher in heterogeneous caves (Mazei et al. 2012). Except for the habitat characteristics, significant differences could be attributed to different methodologies. For example, research in the Grotta del Mulino cave (Italy), that included intensive laboratory experiments, revealed 129 taxa of protists in the collected samples (Luca et al. 2005).

Protists found in the Veternica cave are mostly taxa that have been registered by other researchers in non-cave habitats (i.e. Gittleson and Hoover 1969; Mazei et al. 2012). However, the recently described testate amoeba *Centropyxis bipilata* has been reported from the Veternica cave (Baković et al. 2019), showing that this cave supports potentially interesting assemblages. *C. bipilata* is distributed within caves of the Dinaric karst and the Veternica cave represents the only isolated karst area where this species was found. These findings imply that caves may be an additional habitat for some widely distributed protists or possibly the only habitat for species found exclusively in caves. As protists in general are still scarcely investigated, especially in soil (Foissner 1997), this hypothesis could not be yet confirmed with certainty.

Some taxa, registered in the Veternica cave (Table 1), could indicate variable saprobity at the prospected sites. For example, the ciliates *Cyclidium glaucoma* and *Cinetochilum margaritaceum* that were common in habitats with guano (Fig. 7), were absent in the hygropetric. The presence of taxa indicative for a some higher saprobic index in caves (Coppellotti Krupa and Guidolin 2003) is implying strong influence of guano as one of the factors that is determining taxa composition. On the other hand, site HP is characterized by the absence of naked amoebae, heliozoans, stramenopiles, predatory ciliates and larger taxa of testate amoebae (e.g. *Difflugia oblonga*, *Euglypha tuberculata*) (Table 1). Taxon richness and abundance (Fig. 5; Table 2) were also lower in this habitat. These results are in concordance with the statement that some subterranean habitats are characterized by simple food webs, consisting out of small number of species (Moldovan et al. 2018). Results from the Veternica cave are showing that in the same cave various trophic conditions could be present. Guano in caves is supporting not only microorganisms, but also groups of specialized organisms called guanophiles and guanobionts (Moldovan et al. 2018). As the taxon richness of meiofauna in the Veternica cave was richest in habitats with guano, it could not be excluded that their presence influenced protist taxa composition and abundance, thus making the habitats with guano in the Veternica cave more distinctive than habitats without guano.

It should be noted that the presence or absence of certain species or taxa in the samples cannot always be reliably detected. Testate amoebae are still relatively easy to register and identify from their (usually empty) shells, although here too it is sometimes

not always possible without observing living specimens for their pseudopodia (lobose or filose). Naked amoebae are much more difficult to detect, as they can die or encyst fairly quickly in the material being transported. For this group, wet slides kept in moist chambers for some time can reveal several species. The same goes for heliozoans. At present, heliozoans can only be reliably identified by SEM.

As the Veternica cave has a large number of visitors, they could also impact taxon richness. It is experimentally confirmed that visitors have strong impact on dispersion of microorganisms and seeding of species not typically found in caves (Fernandez-Cortes et al. 2011; Mulec 2014; Mulec et al. 2017). For example, the entrance of the Veternica cave has numerous small water pools that visitors are stepping to before entering the cave. These human vectors of transport could be also attributed to the living protists and especially their encysted stages that are smaller and lighter (i.e. even more easily transferred). Findings of protist cysts in prospected habitats in the Veternica cave imply that some present taxa may have been introduced as cysts.

Results of the nMDS analysis showed a specific grouping within the researched sites (Fig. 6). When comparing the taxon richness and abundance between habitats in this research some variability can be observed (Fig. 4; Table 2). The most distinctive characteristic of HP is a low taxon richness and also a relatively narrow spread of taxon richness over six months of research compared with SP and CP. A low abundance of protists in HP is, in contrast to SP and CP, even more distinct. On two occasions, protists were not detected in HP. Negative results from cave samples were also mentioned by other authors (Delhez and Chardez 1970; Golemansky and Bonnet 1994) implying occasional conditions with low energy input. When these conditions are present, the protists communities are undetectable by the sampling methodology used in this research. Considering that guano was absent in all samples of HP, this habitat, in contrast to habitats SP and CP, is very poor in organic matter, which is probably the cause of low taxon richness and abundance.

The differences between individual sites of habitats SP and CP can be explained by the temporal variability of bat guano (Fig. 7). Research in the Veternica cave corresponded with the period of pre-hibernation and hibernation of the large bat colony, so their droppings found on the researched habitats were of variable age. Taxon richnes was highest on SP-2 (36 taxa) (Table 1; Fig. 7) where guano was of the highest quantity. Research of naked amoebae in the guano habitats of some Slovenian caves showed that their taxon richness is higher in fresh guano deposits, while they were absent in guano deposits that were several decades old (Mulec et al. 2016). Our results are in agreement with the mentioned findings, as the naked amoebae were most diverse at site SP-2 with the highest guano quantities during the whole research period (Fig. 7). Also, naked amoebae have not been recorded in the hygropetric that lacked guano during the whole research period (Table 1).

The modifying effect of guano in the Veternica cave can have several consequences. Bat guano represents a rich food source for protists, thus it increases taxa abundance. This was also reported by some other researchers (e.g. Golemansky and Bonnet 1994). A higher abundance of all organisms (including bacteria) establishes complex food webs that support more taxa. It also means that some potentially rare taxa are more likely to

be discovered. A second impact of guano is the establishment of more diverse microhabitats that can be colonized by protists. Due to the small number of materials present in caves (both organic and inorganic), a limited number of habitats can be formed. The presence of guano in a habitat can increase the heterogeneity in a specific location due to the large number of insect chitin leftovers in the guano that could provide shelter for microorganisms. They serve as an additional microhabitat for some taxa. From the perspective of microorganisms, habitats in caves are more diverse for them than for multicellular organisms. Guano certainly increases this heterogeneity further.

Except for the mentioned energy input to the habitats, the factors that are contributing to the distinction of the habitats showed by mMDS (Fig. 6) could be related to other habitat characteristics. The difference between SP and CP protist assemblages, even though these habitats appear almost similar, can be attributed to differences in sediment on their bottoms. The bottom of SP is made of calcite minerals that create a heterogeneous surface due to the spaces between the mineral grains. These spaces can be used by protists. On the other hand, clay creates a compact and homogenous surface. The low permeability of clay prevents the formation of additional habitats. Clay deposits, as distinctive habitat of protists, was also mentioned by Vandel (1965).

The differences between habitats can also be attributed to their microhydrology. SP and CP are actually small lentic habitats within caves with the ability to held sediments. Retention of sediments, especially bat guano, significantly increases the energy input to these habitats. As already mentioned, higher abundance and taxon richness of protists in habitats with bat guano and cricket excrements has already been observed in caves (Gittleson and Hoover 1970; Golemansky and Bonnet 1994; Mulec et al. 2016). Findings of heliozoans and the large stramenopile *Actinophrys sol* exclusively in SP and CP, indicate conditions closer to surface lentic habitats, even though these habitats are characterized by shallow and very small water pools. The HP, on the other hand, is better described as a lotic habitat as the water seeps constantly on the walls surface, so there is no significant water or sediment retention. Thus, the hygropetric is a food deprived environment compared to SP and CP.

Good synchronicity between the different habitats in the Veternica cave indicates similar seasonal trends in diversity and abundance of protists. As the Veternica cave microclimate showed relatively small variations and considering the constant absence of light, the cause of similar seasonal trends could be a reflection of the hydro-meteorological conditions in the cave drainage area. The water coming from the surface transports organic matter to the cave ecosystems (Simon et al. 2007) which affect the subterranean food webs. The connection between discharge events and diversity and abundance of microorganisms has already been noted in karst aquifers (Wilhartitz et al. 2013; Benk et al. 2019). The impact of hydro-meteorological conditions on protist assemblages in the Veternica cave (that was performed on the same dataset as this research), was also confirmed by Kajtezović and Rubinić (2013) using the regression tree algorithm. Thus, it can be concluded that surface hydro-meteorological conditions have a relatively homogenized impact on the subterranean protist assemblages in the prospected habitats in the Veternica cave. As the significant differences in spatio-

temporal taxon richness and abundance in habitats existed, despite the seasonal synchronicity between habitats, it can be assumed that protist assemblages in the Veternica cave are shaped both by hydro-meteorological conditions in the cave drainage area together with already present conditions in the cave.

Conclusion

This research presents one of the rare studies of spatio-temporal taxon richness and abundance of protist assemblages in karst caves. Analysis, based on six months research, showed that statistically a significant difference between sinter and clay pools habitats and hygropetric habitats exist. This result is implying that cave habitats, holding very small quantities of water, are much more diverse than previously thought.

The recorded taxon richness of protists in the Veternica cave shows a high number of taxa, especially considering the small number of samples and prospected habitats. Thus, taxon richness of protists in caves could be much richer in comparison with diversity of cave animals. As protists are very scarcely researched in caves, future research should focus on investigating characteristics of small aquatic habitats in conditions where bat's guano is absent in order to determine its impact on protist assemblages.

Research in the Veternica cave provided valuable information about the protists in caves that are an important component of subterranean food webs supporting stunning biodiversity of subterranean animals. Thus, further effort should be given to the research that could shed light on these trophic relations.

Acknowledgements

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Comparative phylogeography of two troglobitic Coleoptera (Leiodidae, Leptodirini) species from Romania based on mitochondrial DNA

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Abstract

About 50 species of cave-obligate Leptodirini (Leiodidae) beetles have been described so far in Romania, most of them populating caves in the Apuseni Mountains (north-western Romania) and the Southern Carpathians. In this contribution, we present the first molecular phylogeographic study of the two troglobiotic *Pholeuon* species from the Apuseni Mountains. The two species are *Pholeuon* (s.str.) *leptodirum* and *Pholeuon* (*Parapholeuon*) *gracile*, endemic to Bihorului Mountains and Pădurea Craiului Mountains, respectively. To examine the genetic divergence within and between the two species we sequenced 571 bp of the mitochondrial COI gene in a total of 145 specimens, 56 specimens of the first species (collected in five caves) and 89 of the second species (collected in eight caves) across their geographic ranges. We found very low genetic variation, four haplotypes in *P. leptodirum* and seven haplotypes in *P. gracile*, and a maximum of 0.7% and 0.9% intraspecific divergence, respectively. However, a significant genetic divergence of 6.55% was found between species. The results are consistent with previous definitions of the two species based on morphological characters, while caution should be taken

* These authors had an equal contribution.

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in considering attributions to different subspecies. Our research contributes to the phylogeographic information of troglobitic beetles, providing a solid basis for future comparison with other terrestrial or aquatic cave adapted species.

Keywords
Carpathians, cave beetles, cytochrome oxidase gene I, *Pholeuon*, population genetics

Introduction

A detailed knowledge of the biology and ecology of the species and their genetic structure at the population level plays a crucial role in minimizing the effect of loss of biodiversity. Important target of global conservation efforts are endemic species. These, with their specific climatic and environmental requirements and generally limited dispersal capacity, are particularly vulnerable to extinction (Myers et al. 2000; Lamoreux et al 2006). Obligate subterranean species, cave-adapted so called troglobionts, are particularly vulnerable to the pollution produced on the surface, which percolates soil and layers of limestone, contaminating the subterranean habitats (Wood and Perkins 2002; Manenti et al. 2021). Subterranean environments are inhabited by a specialized fauna living in relatively stable conditions that creates a unique biological laboratory where evolutionary and ecological processes can be studied in situ (Mammola 2019). Subterranean terrestrial habitats are characterized by absence of light, saturated air humidity, constant temperatures and often scarce food resources (Howarth et al. 2008). The ecological balance is, therefore, fragile and any disturbance can potentially cause alteration of the natural conditions, fragmentation of the habitat and populations, with the possible extinction of the troglobitic species.

Romania hosts many unique karst landscapes and caves, and several types of endemism are found in its subterranean fauna. The occurrence and distribution of the Romanian cave fauna can be to a larger extent explained by paleogeography and ecology of the group (Moldovan 2008). Among them, the Leptodirini (Coleoptera, Leiodidae) species include strictly cave-adapted beetles and represent a conspicuous group of species endemic to one or a few caves in a karst area. Indeed, eight Leptodirini genera with 49 endemic species inhabit Romanian caves (Moldovan et al. 2020). Their limited distribution in a fragmented karst landscape make Romanian Leptodirini a model group for studying speciation and historical biogeographic processes both at fine and large scales. Molecular studies on Leptodirini species are still very limited in number, and the most comprehensive study was undertaken by Ribera et al. (2010); this comprised 57 Leptodirini species from all the major lineages distributed in the Western Mediterranean area, including two Romanian species. The molecular clock approximation suggested that the main Western Mediterranean lineages originated in Early-Mid Oligocene and that the ancestral species were already present in the geographical areas in which they are found today (Ribera et al. 2010).

Romanian Leptodirini group is considered to derive from the Dinaric ancestors, before the separation of the Carpathian Mountains from the Dinarides (Jeannel 1931; Decu

and Negrea 1969; Moldovan and Rajka 2007). The Romanian Carpathians are divided into three geographical units: The Apuseni Mountains (North-West), the Eastern Carpathians, and the Southern Carpathians. The Apuseni Mountains are characterized by an amazing variety of relief micro forms, both above and underground, and a considerable number of caves (3,960 caves, with a cave density of 3.5 caves/km^2) (Cocean 2000). The unique features of the fragmented karst in this region, interrelated with the underground hydrological network, could present a great opportunity for the dispersal of the species. Whereas the complex geological settings, from local to regional context, potentially function as geographical barriers for the dispersal of subterranean fauna (Moldovan 2008).

Romanian Leptodirini were studied, so far, for various aspects: ecological, taxonomic, either by classical taxonomy (the revision of genus *Drimeotus*; Moldovan 2000) and by morphometrics (Racoviță 1996; Racoviță 1998–1999; Racoviță 2010, 2011), historical biogeography (Moldovan and Rajka 2007), cytogenetic (Buzilă and Marec 2000), molecular phylogeography (Bucur et al. 2003). Studies on molecular phylogeography of Leptodirini were carried out on the phyletic series of *Drimeotus* with three genera, *Drimeotus*, *Pholeuon*, and *Protopholeuon*, all three endemics to Apuseni Mountains (Bucur et al. 2003). *Drimeotus* and *Pholeuon* include three and two subgenera, respectively, while *Protopholeuon* is a monospecific genus. Species belonging to *Pholeuon* and *Protopholeuon* are considered more troglomorphic (sensu Christiansen 2012) (longer appendages, slender body) than *Drimeotus* (Moldovan et al. 2007). The study of Ribera et al. (2010), including representatives from Romanian Carpathians, established the monophyly of the phyletic series *Drimeotus*. On the contrary, the subgenera belonging to *Drimeotus* and *Pholeuon* genera were not monophyletic (Bucur et al. 2003), offering multiple independent colonization events by surface ancestors as a possible explanation for their actual distribution.

In the present study we carried out a comparative phylogeographic analysis of the two cave adapted Leptodirini subgenera from the Apuseni Mountains. In particular, we considered two mountain ranges, Pădurea Craiului and Bihorului, with the species belonging to their corresponding endemic subgenera: *Pholeuon* (*Parapholeuon*) and *Pholeuon* (s. str). Until present, three species have been described from each subgenus, *Parapholeuon* with *P. gracile*, *P. moczaryi*, and *P. angustiventre* and *Pholeoun* s. str. with *P. angusticolle*, *P. knirschi* and *P. leptodirum.* For this study, one species from each subgenus, *P. gracile* and *P. leptodirum*, both including several populations/subspecies, were considered. The analyses included all three described subspecies of *P. gracile* while only five of eleven described subspecies of *P. leptodirum* were included (Racoviță 2011). The description of subspecies is rather controversial as it is solely based on morphometric characters, and their taxonomical status reaches beyond the scope of this paper.

The main aims of the present study were: i) to test the congruence between the subspecies identified on the basis of morphometric measurements and the putative molecular species delimited using DNA barcoding (mitochondrial cytochrome oxidase subunit I, COI); and ii) to investigate the degree of genetic differentiation both within and between the two cave-adapted species, and to analyse it according to the geographic distribution of populations.

Materials and methods

All analyzed populations are endemic in two massifs of the Apuseni Mountains, Bihorului and Pădurea Craiului, and have limited distribution in one or few caves only (Fig. 1 and Table 1).

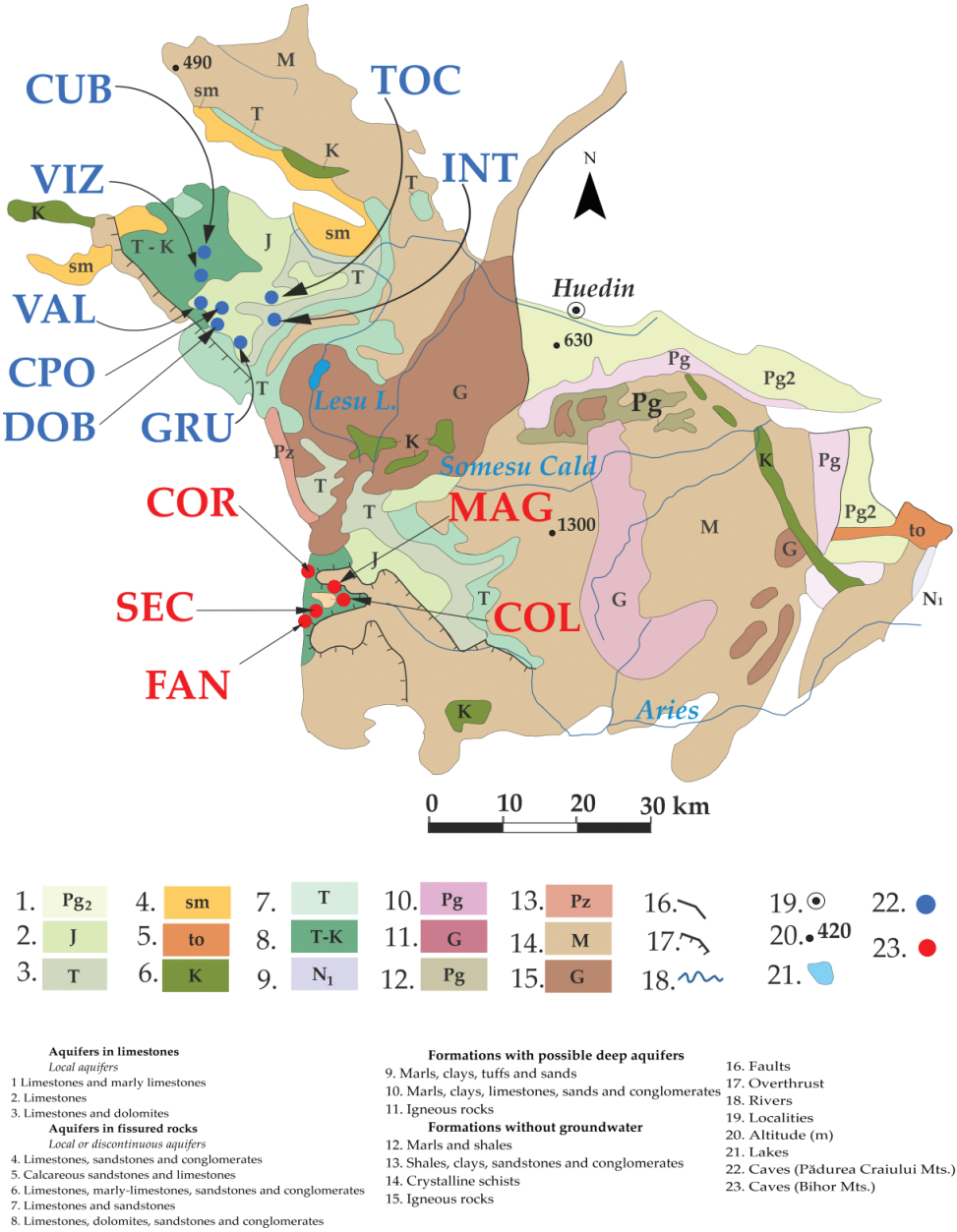

Figure 1. Sampling sites of *P.* (*Parapholeuon*) *gracile* in Pădurea Craiului Mountains (blue) and *P.* (s. str.) *leptodirum* in Bihorului Mountains (red). Codes refer to the caves' name, as indicated in Table 1.

Table 1. Leptodirini subspecies (as proposed by Racoviță 2011) included in the study with the codes used throughout the paper, the hydro-karstic basins, altitude and slope where the caves are located. Sample size (N), haplotypes and number of specimens sharing each haplotype are also indicated.

Cave Name	Code	River valley	Basin	Subspecies	Altitude (m a.s.l.)	Geographic slope	N	Haplotype (no. specimens)
Pădurea Craiului Mountains – *P.* (*Parapholeuon*) *gracile*								
Cubleș	CUB	Vida	Holod	*P. g. gracile*	440	right	11	H1 (9), H2 (2)
Vizu II	VIZ	Vida	Holod	*P. g. bokorianum*	350	left	12	H3 (5), H6 (6), H5 (1)
Tocoș	TOC	Runcșor	Roșia	*P. g. chappuisi*	585	right	11	H1 (8), H2 (2), H7 (1)
Întorsuri	INT	Runcșor	Roșia	*P. g. chappuisi*	575	right	11	H1 (9), H2 (2)
Ciur Ponor	CPO	Albioara	Roșia	*P. g. chappuisi*	510	left	10	H3 (10)
Doboș	DOB	Albioara	Roșia	*P. g. chappuisi*	465	left	11	H3 (11)
Vălău	VAL	Albioara	Roșia	*P. g. chappuisi*	355	right	12	H3 (9), H4 (3)
Gruieț	GRU	Șteazelor	Roșia	*P. g. chappuisi*	300	left	11	H1 (9), H2 (2)
Bihorului Mountains – P. (s.str.) leptodirum								
Coliboaia	COL	Sighiștel	Sighiștel	*P. l. jeanneli*	560	right	11	H8 (11)
Măgura	MAG	Sighiștel	Sighiștel	*P. l. hazayi*	550	right	12	H8 (12)
Corbasca	COR	Sighiștel	Sighiștel	*P. l. moldovani*	500	left	11	H8 (6), H9 (5)
Fânațe	FAN	Bulzului	Crișul Băița	*P. l. leptodirum*	560	right	10	H8 (8), H11 (2)
Secătura	SEC	Bulzului	Crișul Băița	*P. l. problematicus*	1080	right	12	H10 (12)

Sampling

Specimens of *P. gracile*, belonging to the three described subspecies (*P. gracile* s. str., *P. g. chappuisi*, and *P. g. bokorianum*), were collected from eight caves located in four different valleys of Pădurea Craiului Mountains (Table 1, Fig. 1). Five populations of *P. leptodirum* (*P. leptodirum* s. str., *P. l. jeannely*, *P. l. hazayi*, *P. l. moldovani*, *P. l. leptodirum* and *P. l. problematicus*) were collected in two different valleys in Bihorului Mountains (Table 1, Fig. 1).

In each cave, between 10 and 12 individuals were collected for the analysis. The total number of specimens was 145 (89 individuals for the first species and 56 for the second one). Specimens were preserved in 95% ethanol until DNA extraction was processed.

DNA extraction, PCR and sequencing

Total genomic DNA was extracted from the entire specimens using the DNeasy Blood and Tissue Kit (Qiagen), following the producer's protocol. A fragment of 571 base pairs (bp) of the mitochondrial Cytochrome Oxidase I (COI) gene was amplified using LCO1490 and HCO2198 primers (Folmer et al. 1994).

Double stranded amplifications were performed in a 50 µl reaction volume containing buffer, 5 µl dNTP's 10 mM, 0.5 µl primer 10 mM (each primer), 0.4 µl TAQ polymerase (5U/µl) and 38.6 µl purified water. Each PCR cycle (of a total of 30 cycles) consisted of a denaturation step at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 7 min. PCR products were purified following the manufacturer's protocol for the PCR-Nucleospin Gel and PCR Clean-Up (Macherey-Nagel). Both strands were sequenced on an automated sequencer.

Genetic analyses

Sequences were aligned and edited with BioEdit (v. 7.2), the number of transitions and transversions were analysed with DNAsp (v 5.10.1) (Librado and Rozas 2009). We used MEGA7 (v.7.0) (Kumar et al. 2016) to analyse interspecific haplotype diversity. Genetic structure between populations of the two species or within populations of the same species was analysed with F-statistics using Arlequin (Excoffier et al. 2010) by calculating the following parameters: haplotype diversity (*h*), the absolute haplotype frequencies, and the nucleotide diversity (π_n). The minimum spanning network was built using PopArt software (Leigh and Bryant 2015).

Mean pairwise intra- and interspecific distances were determined using MEGA (Tamura et al. 2013). Analysis was conducted using uncorrelated p-distance. The analysis involved 145 nucleotide sequences. All codon positions were included.

Using PAST software (Hammer et al. 2001) a Mantel test (Mantel 1967) with 5,000 simulations was carried out to test for an isolation-by-distance (IBD) signature (a positive correlation between geographic and genetic distances (Wright 1943; Slatkin 1993).

The hierarchical distribution of genetic variation was characterized using analysis of molecular variance (AMOVA). This method apportions genetic variation within and among groups, estimating Φ-statistics (Weir and Cockerham 1984; Excoffier et al. 1992; Weir 1996) that are analogous to Wright's hierarchical fixation indices (FST) under the island model of gene flow (Wright 1951). Three-level AMOVA was conducted in ARLEQUIN 3.5.1.2 (Excoffier et al. 1992, 2005) using an FST-like estimator (Fixation Index). For each of the two considered species, AMOVA was run three times considering different groups of populations on the basis of the geological and geographic characteristics. In particular, samples were partitioned by the river basin (Table 1), populations within geographic regions and inside each population. The tests included permutation of inferred haplotypes among groups (FCT); individual haplotypes among populations but within group (FSC); inferred haplotypes among populations (FST).

In order to test for the monophyly of the two Leptodirini species we carried out phylogenetic analysis within a Bayesian framework. J model test (Dariba et al. 2012) was used to perform a hierarchical likelihood ratio test and calculate approximate Akaike Information Criterion (AIC) values of the nucleotide substitution models.

Phylogenetic analysis was performed using Bayesian inferences as implemented by the software MrBayes 3.2.7 (Ronquist et al. 2012). Two simultaneous searches, comprising four Markov chains (MCMC) each and starting from a randomly chosen tree were run for 1,000,000 generations and sampled every 100 generations.

Convergence on a common phylogenetic topology by separate Bayesian searches was checked using Tracer 1.7 (Rambaut et al. 2018). The effective sample size (ESS) of all parameters showed values above 1,000 (values much higher than the threshold of statistical significance, ESS > 200) in both simultaneous searches, indicating that MCMC had converged. Out of 20,000 trees, the first 1,000 were discarded as burn-in, and posterior probabilities (PP) were calculated from post-burn-in trees. The tree was rooted with a species of Leptodirini from Sardinia, *Ovobathysciola* sp.; we were provided with one specimen.

Results

Sequences were obtained from a total of 145 individuals, 89 for *P. gracile* and 56 for *P. leptodirum* (Table 1).

The alignment consisted of 571 bp and defined 46 variable sites, of which 44 were parsimony informative. The nucleotide diversity among all sequences was π_n = 0.033. The sequences are deposited in Genbank with the Accession Numbers OL457148–OL457159 (for H1–H11 and *Ovobathysciola*, respectively).

Intraspecific variability

We identified seven haplotypes for *P. gracile* (numbered H1–H7; Table 1), separated from each other by a maximum of eight mutations, whose geographic distribution is represented in Fig. 2. The haplotypes were separated in two haplogroups according to the genetic divergence between them. One group consisted of specimens of Doboş (DOB), Valău (VAL), Ciur-Ponor (CPO) from Albioara Valley/Sohodol basin, and Vizu II (VIZ) from Vida Valley/Holod basin. The second group was represented by Cubleş (CUB) from Vida Valley/Holod basin, Tocoş (TOC)/Sohodol basin, Întorsuri (INT) from Runcşor Valley/Runcşor basin, and Gruieţ (GRU) from Şteazelor Valley/Roşia basin.

The most widespread haplotypes were H1 and H3, each showing a frequency of 40% in all the analyzed specimens. In particular, individuals from Ciur-Ponor and Doboş caves were fixed for H3 haplotype that was also identified in two other caves, Valău (75%) and Vizu II (42%). Haplotype H1 was shared by the individuals from Cubleş (82%), Gruieţ (82%), Întorsuri (82%), and Tocoş (73%) caves. Haplotypes H5 and H7 were identified in a single specimen, each from Vizu II and Tocoş caves, respectively.

Haplotypes H6 and H4, present with a frequency of 50% and 25%, appeared to be exclusive of Vizu II and Vălău caves, respectively. Haplotype H2 was spread in four caves, Cubleş, Gruieţ, Întorsuri, and Tocoş, with a frequency of 18% in each case. The haplotype diversity for *P. gracile* was *Hd* = 0.684 and nucleotide diversity was π_n = 0.003.

The haplotype network for *P. gracile* is illustrated in Fig. 3a. The identified haplotypes are divided in two clusters: one comprising haplotypes H1, H2 and H7, and

Table 2. *P.* (*Parapholeuon*) *gracile* – computing conventional FST from haplotype frequencies (values in bold indicate significance at the 0.05 level after Bonferroni correction).

	CUB	VIZ	TOC	INT	CPO	DOB	VAL
VIZ	**0.52155**						
TOC	-0.07556	**0.45111**					
INT	-0.10000	**0.52155**	-0.07556				
CPO	**0.82896**	**0.43893**	**0.75370**	**0.82896**			
DOB	**0.83636**	**0.45372**	**0.76364**	-0.10000	0.00000		
VAL	**0.63047**	**0.25069**	**0.56005**	**0.63047**	0.15691	**0.63047**	
GRU	-0.10000	**0.52155**	-0.07556	-0.07556	**0.82896**	**0.83636**	**0.63047**

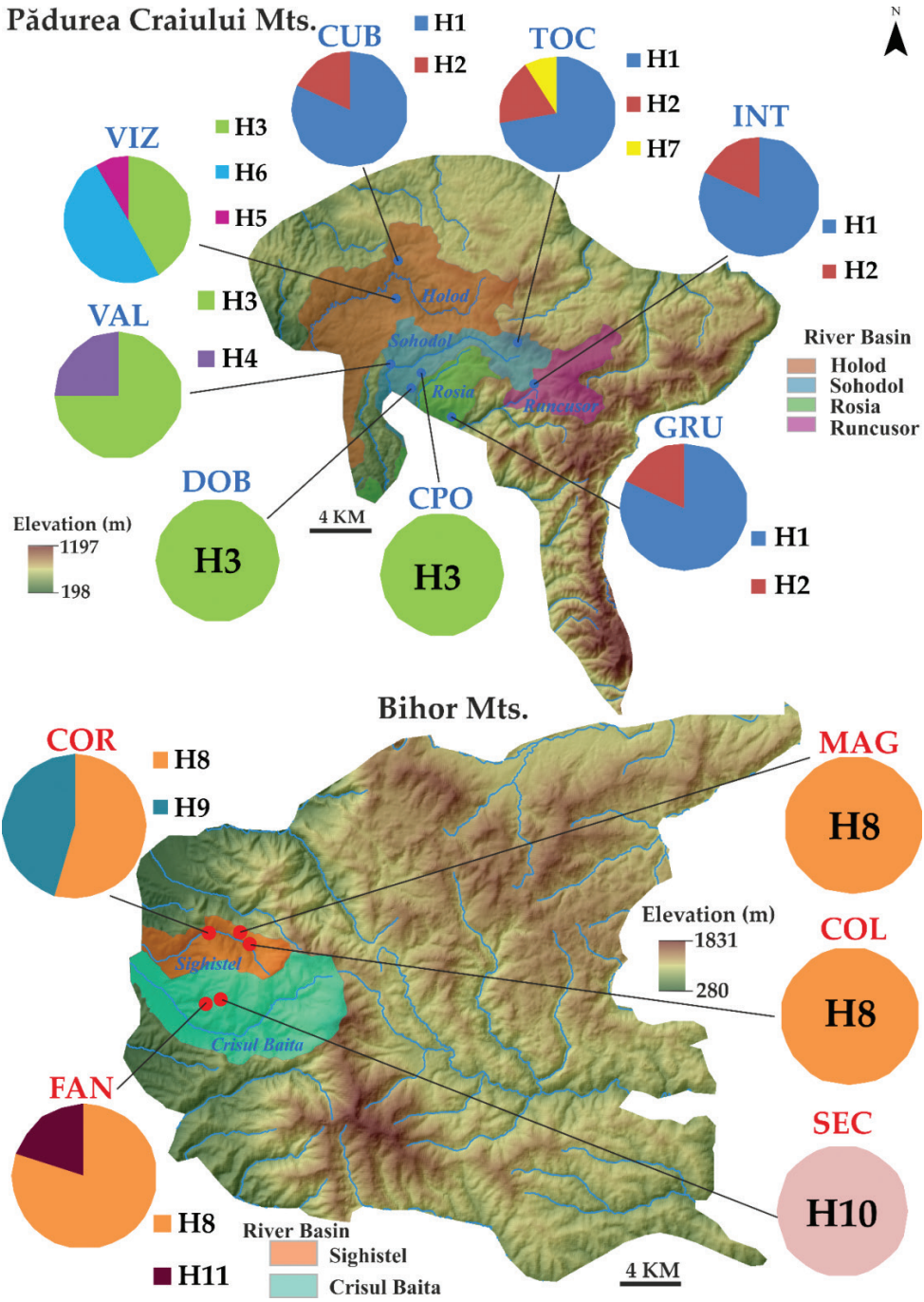

Figure 2. Geographic distribution of haplotypes in *P. gracile and P. leptodirum*. Individuals of DOB and CPO caves are fixed for the haplotype H3 in *P. gracile*; individuals of SEC cave are fixed for the unique haplotype H10 in *P. leptodirum* (abbreviations for the names of caves as in Table 1).

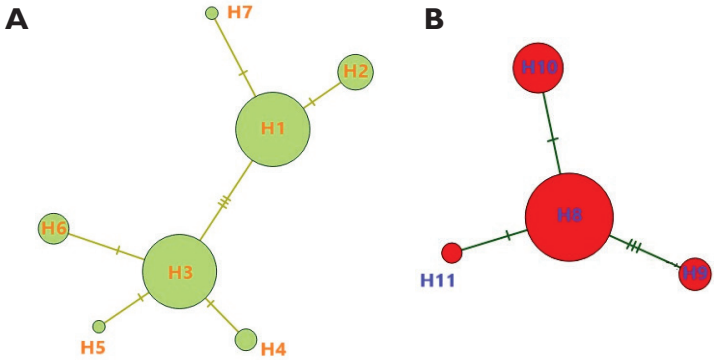

Figure 3. Minimum spanning networks **a** for *P. gracile* with haplotypes H1–H7 and **b** for *P. leptodirum* with haplotypes H8–H11. Multiple mutational steps between haplotypes H1–H3 and H8–H9 could be either un-sampled haplotypes or extinct ones. Haplotype numbers are as in Table 1.

the other haplotypes H3–H6. The two clusters are differentiated by three mutations. Within each cluster, the haplotypes are separated by only one mutation.

FST values for *P. gracile* are presented in Table 2, in which 19 of 28 inter-populational comparisons provided significance at 0.05 level of probability. All negative values indicated a lack of genetic differentiation between the respective populations. FST values over 0.80 indicated a certain degree of genetic differentiation and reduced gene flow between populations included in the two clusters. Mean p-genetic uncorrelated distances between populations belonging to *P. gracile* ranged between 0.1 and 0.9%.

Analysis of molecular variance (AMOVA), suggested some degree of genetic structure within each population (FST = 0.856, P = 0). Genetic variation among different geographic groups and among populations within each clade was 49.4% and 36.2%, with FCT = 0.493 (P > 0.05) and FSC = 0.715 (P = 0), respectively. Mantel test did not suggest a clear isolation by distance across the sampled region (R2 = -0.002, P > 0.05).

In *P. leptodirum* only four haplotypes have been identified (numbered H8–H11; Table 1), separated from each other by a maximum of five mutations, whose geographic distribution is shown in Fig. 2. The most frequent haplotype was H8, spread in all the analyzed cave populations, except for Secătura Cave. In this case, all sampled individuals were fixed for haplotype H10. In particular, samples from Coliboaia and Măgura caves were fixed for haplotype H8 that was also identified in Fânațe (80%) and in Corbasca (55%) caves. Moreover, Fânaţe and Corbasca caves showed also haplotype H11 and H9 with a frequency of 20% and 45%, respectively. The haplotype diversity for *P. leptodirum* is *Hd* = 0.517 and nucleotide diversity was π_n = 0.001. The haplotype network for this species is illustrated in Fig. 3b.

For the genetic structure of *P. leptodirum*, the indicators of genetic population structure (FST) are presented in Table 3; of 10 inter-populational comparisons, 7 provided significance at the 0.05 level of probability. The highest FST values (>0.70) were for comparisons between the population from Secătura Cave with the other four

Table 3. *P.* (s. str.) *leptodirum* – computing conventional FST from haplotype frequencies (values in bold indicate significance at the 0.05 level after Bonferroni correction).

	COL	MAG	COR	FAN	SEC
MAG	0.00000				
COR	**0.40000**	**0.41463**			
FAN	0.12438	0.13669	**0.19767**		
SEC	**1.00000**	**1.00000**	**0.73742**	**0.83762**	

populations, indicating a degree of genetic differentiation between populations and a limited level of gene flow.

Mean genetic distance between populations belonging to *P. leptodirum* ranged from 0.1 to 0.7%.

Analysis of molecular variance (AMOVA) suggested, also in this case, some degree of genetic structure within the populations (FST = 0.623, P = 0). Genetic variation among different geographic groups and among populations within each group was 11.9% and 50.4%, with FCT = 0.119 (P > 0.05) and FSC = 0.572 (P = 0), respectively. Mantel test did not suggest a clear isolation by distance across the sampled region (R2 = -0.002, P > 0.05).

Interspecific variability

As expected, genetic variation between *P. gracile* and *P. leptodirum* was much greater than intraspecific variation, with a mean genetic distance of 6.55%. Analysis of molecular variance (AMOVA) carried out considering the taxonomic assignment of each population and not the valley, suggested that the two species are well differentiated showing a genetic variation of 95.6%, with FCT = 0.955 (P = 0.002).

The phylogenetic analysis, carried out considering TrN + G model, as suggested by J model test, strongly supports the monophyly of the two species and their clear genetic separation. *P. leptodirum* showed a certain degree of homogenization among its populations, although each was described as a different subspecies. On the other hand, *P. gracile* showed a higher genetic divergence between the analyzed populations, forming two distinct clades. However, in this case the subspecies were not genetically supported, since Cubleș (*P. g. gracile*) and Vizu (*P. g. bokorianum*) did not form different clades, but are linked to the other populations, representing *P. g. chappuisi* (Fig. 4).

Discussion

The genetic variability at COI DNA barcode detected in populations of both analyzed species of *Pholeuon* is quite low. We found a maximum of eight mutations between haplotypes of *P. gracile* and a maximum of five mutations separating the haplotypes of *P. leptodirum*. This result agrees with other studies concerning the analysis of COI DNA barcode in cave dwelling species. Intraspecific genetic variation in seven

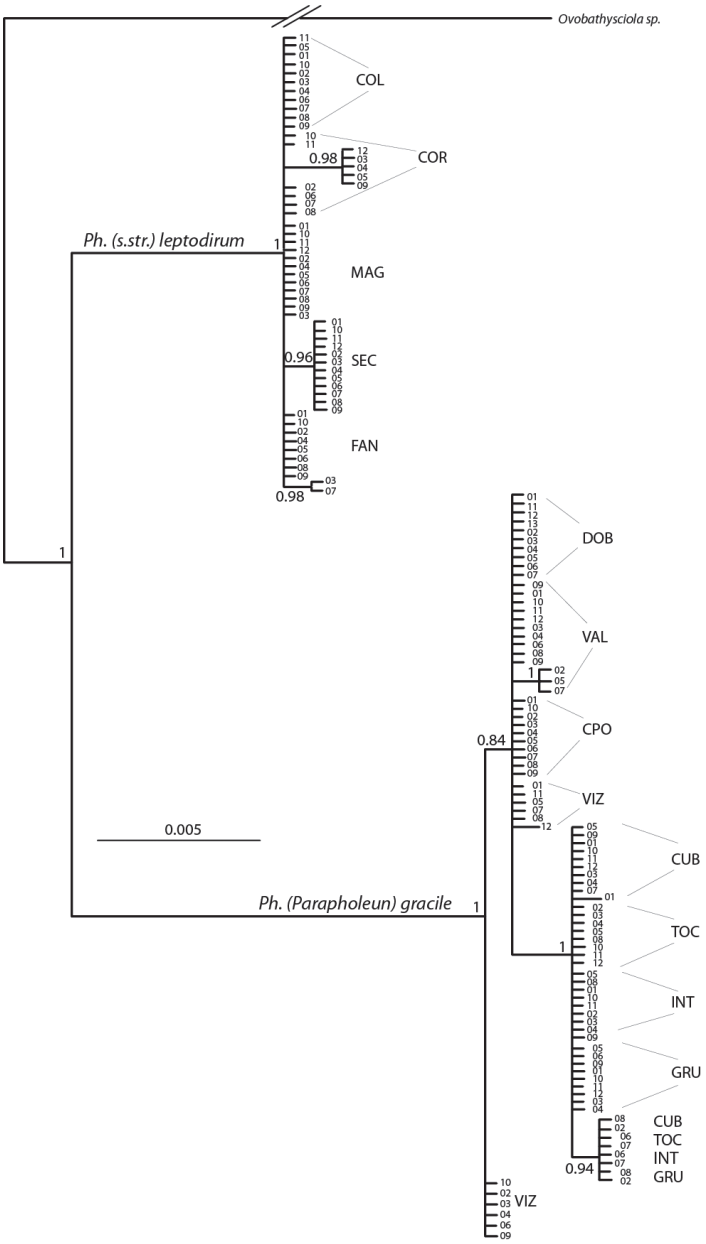

Figure 4. Bayesian tree constructed from 145 individuals of *P. gracile* and *P. leptodirum* from the Apuseni Mountains, belonging to 13 populations (caves). The genetic separation of the two species is clear. As outgroup an *Ovobathysciola* sp. from Sardinia was used.

species of *Bathysciola* (Coleoptera, Leiodidae, Leptodirini) from Central-Southern Italian Apennines and Pre-Apennines ranged from 0 to 1.5% (Latella et al. 2017), while in *Dolichopoda* cave crickets (Orthoptera, Rhaphidophoridae) intraspecific genetic variation ranged from 0 to 1% (Allegrucci et al. 2005, 2014, 2021). The investigation

of two species of the *Tetracion* troglobitic millipede (Diplopoda, Callipodida, Abacionidae) revealed a maximum of 1.4% intraspecific genetic divergence (Loria et al. 2011). Also, populations' COI genetic divergence levels in the troglobitic *Darlingtonea kentuckensis* (Coleoptera, Carabidae, Trechinae) were found at 1.3% (Boyd et al. 2020).

However, despite the low variability, the studied cave populations showed a significant level of genetic structure. AMOVA analysis evidenced significant partitioning of variation within and among populations in both studied species. This result is not surprising for troglobionts because it reflects the possible barriers between the different caves and/or groups of caves to which populations are confined. On the other hand, genetic variation is not significantly partitioned among geographic groups in both species (FCT shows $P > 0.05$ in both cases) and Mantel test did not show a phylogeographic pattern. These results could be explained by the evolution of caves both in Pădurea Craiului Mountains inhabited by *P. gracile* and in Bihorului Mountains where *P. leptodirum* is found.

The formation of the caves is strongly related to hydrological network development and the tectonics, both at regional and local scales. The area in Pădurea Craiului Mountains where the caves are located is a highly tectonic region (Orașeanu 2020), with several stages of cave systems evolution. Caves can evolve quite differently from one another regarding water input, rock type, geotectonic features, and local hydrological system although being part of the same basin (Rusu 1981).

The hydrographic network of Pădurea Craiului Mountains is not well organized due to very intense processes of karstic caption (Orășeanu 2020) that promotes the formation of geological and hydrographic barriers, at the same time preventing gene flow between cave populations. For example, Vizu and Cubleş caves, on one hand, and Doboş/Ciur Ponor and Valău caves, on the other hand, are located in the same valley, but on the opposite slopes. Vizu Cave population showed a haplotypic composition (H3, H5, H6) completely different from Cubleș Cave (H1 and H2). The two caves are located on different slopes of the same valley, less than 1 km apart from each other. Cubleş and Vizu caves could belong to different stages of evolution and development of the Vida River (Orășeanu 2020). Both of them were carved by tributary streams (Cubleş by Blajul and Vizu by Viduța), controlled by the local water table (e.g., different incision rates). Because the relative altitude varies for the two caves, with Cubleş at ~50 m and Vizu at ~2 m above the present waterflow the river Viduța might act as a hydrographical barrier even during the incision of the valley and, therefore, could promote genetic isolation and differentiation of populations. We cannot assume that the paleogeographic changes were the only factors, but we can hypothesise that these could be the first step towards the isolation of populations. Ciur Ponor and Doboș caves are located on the same side of Albioara valley, specimens of the two populations share the same haplotype, H3. In Vălău cave population, located on the opposite slope, haplotype H3, but also the unique haplotype H4 have been identified. Since, in both cases, caves differ in aplotype composition, the rivers could act as hydrographic barriers, preventing the dispersal of populations. On the contrary, Tocoș, Întorsuri and Gruieț populations share haplotypes, as no hydrographic barrier separates these caves (Table 1, Fig. 2).

Bihorului Mountains, with caves hosting *P. leptodirum*, are mostly comprised by limestones, dolomites, conglomerates, and eruptive rock (Seghedi 2004). Still, the

development of the karst network in Bihorului Mountains is characterized by intense fragmentation of the carbonate rocks with the development of large-scale karst systems and a petrographic mosaic shaping the relief. Populations of Măgura/Coliboaia caves, located on the same side of the valley, share the same haplotype (H8). At the same time, specimens from the Corbasca cave, located on the opposite slope, have the haplotype H8 and the unique haplotype H9. The populations from Fânațe and Secătura caves are situated on the same side of Bulzului valley. The caves formed in the same type of limestone are geographically close (~1 km). The first cave is inhabited by *P. l. leptodirum*, while the second one by *P. l. problematicus*. Their haplotypic composition is completely different, with Fânațe Cave population showing the most common haplotype H8 and the unique haplotype H11 in low frequency, while Secătura Cave population is fixed for the unique haplotype H10, suggesting a complete lack of gene flow. Although the two caves have certain similarities and are located at different altitudes (Secătura at 740 m.a.s.l. and Fânațe at 580 m.a.s.l.), they could have been populated at different stages by the hypothetical ancestral populations that were already genetically differentiated.

All these local features could establish geologic and hydrogeologic barriers, even for caves that are geographically close. So, even the smallest change at a certain time, in the local evolution of a cave could, be a limiting factor for the dispersal of cave populations (Sánchez-Fernández et al. 2018). Different slopes of the same river valley could represent a geographic barrier strong enough to hamper gene flow in species with poor dispersal capabilities. This could potentially be even reinforced when unsuitable habitats (i.e. impermeable strata, dry conditions, small voids etc) must be crossed. This assumption needs to be validated with additional analyses conducted on a more extensive sampling design, that would include multiple basins with multiple caves on the opposing valley slopes.

In conclusion, in both of the analyzed species, *P. gracile* and *P. leptodirum*, the genetic divergence of the COI DNA is too low to discriminate between different subspecies, although in some cases a certain degree of intraspecific genetic structure has been found (for example, between the proposed subspecies *P. g. bokorianum* and *P. l. problematicus*), suggesting that a reassessment of their status is needed. This result was expected because the genetic divergence at DNA barcoding is not informative about the species status when recently diverged species are compared and complete lineage sorting has not yet been achieved (DeQueiroz 2005; Lencioni et al. 2021). Episodes of gene flow between the different proposed subspecies represent a possible explanation preventing complete lineage sorting and most of the populations belonging to the two considered species here are not completely isolated. Moreover, the identification of species based on one single genetic marker can be incongruent with species identification using morphological characters (Moritz and Cicero 2004; Matz and Nielson 2005; Allegrucci et al. 2014; Lencioni et al. 2021).

In the Bayesian analysis of the relationships between the analyzed taxa, the two *Pholeuon* species are monophyletic and well differentiated from each other, with *P. gracile* showing a higher differentiation than *P. leptodirum*. As far as interspecific variation is concerned, the mean genetic distance was between 6.4 and 7.7%, with a mean value of 6.55%. Following Hebert et al. (2003) COI divergence ranges from

below 1% to 16–32% between species of beetles within the same genus with an average sequence divergence of 11.2. Our value falls within the intermediate range, suggesting that the two species are well differentiated and possibly for a long time. It is not rare to find high genetic differentiation between species of troglobionts as a result of allopatric speciation and the formation of hydro-geographic barriers due to changes in the surface landscape along the different climatic periods. Leptodirini appears to be an ancient cave group, as demonstrated from previous papers (Caccone and Sbordoni 2001; Ribera et al. 2010; Latella et al. 2017) with different species separated for long periods of time and accumulating genetic divergence. High genetic differentiation has been found also in a group of nine species of *Bathysciola*, where the interspecific genetic distances ranged from 3.1% of up to 15.1% (Latella et al. 2017). In this case, the two main lineages of *Bathysciola* were considered and divergence times suggested a Miocene deep cladogenesis. The two clades were shaped by the geological events during the Pliocene and the climatic changes of the Pleistocene (Latella et al. 2017). On the other hand, low levels of mitochondrial diversity have been found in species of the *Pholeuon* genus belonging to *Drimeotus* phyletic lineage although they showed deep cladogenesis with species belonging to the *Drimeotus* genus, included in the same phyletic lineage and revealing a possible split in the late Miocene (Bucur et al. 2003).

In conclusion, based on the observed genetic structure between the different populations of the two *Pholeuon* species further studies including more populations and species are needed to understand the genetic variation patterns of the group and provide valuable information for the life histories and conservation of Leptodirini in Romania. The data presented in this contribution – albeit preliminary in terms of sampling and limited in terms of genetic markers– confirm the importance of subterranean environment as a reservoir of biodiversity at a microgeographical scale. Such biodiversity should be hence managed accordingly.

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Bat fauna and conservation assessment of Kurdistan caves, Iran

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Abstract

The populations of cave-dwelling bat species are encountering a remarkable decline all over the world. To plan effective conservation projects for bats and their cave roosts, collecting data on their distribution in a particular region is essential. Furthermore, developing an applicable index that incorporates both biotic and abiotic parameters relevant to caves is useful to prioritize caves for conservation management. Recently, there has been a growing interest in studying bat fauna of Iran. The Kurdistan province in west of the country is entirely mountainous, having a suitable geological substratum for formation of caves. Previously, five bat species were reported from Kurdistan. The current study has improved the data by doubling the number of cave-dwelling bat species of the province. A total number of 61 records of 10 species were documented. Overall, of 28 caves studied, 26 caves were used by bats. Each cave hosted one to six bat species. To prioritize Kurdistan caves for conservation programs, the Bat Cave Vulnerability Index (BCVI) was employed for 26 caves explored in this study. Four caves, including Karaftu, Kamtaran, Darvish Ouliya, and Kouna Sham-Sham, were highlighted due to their highest BCVI value. The other 22 caves received medium or low priority values. In the current study, we provided data on the bat fauna of Kurdistan caves, in addition to evaluate their conservation priorities by applying an assessing index for the first time in the country.

Keywords

Bat, BCVI, cave, conservation priority, fauna, Kurdistan province

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Introduction

Caves are considered as appropriate roosts that provide a relatively cool and stable environment for many organisms, including bats. Some bat species use caves only for hibernacula, while others dwell in caves year-round using them also as mating and nursery, as well as day roosts (McCracken 1989). Therefore, the survival of many bat species depends on the presence of natural caves (Mickleburgh et al. 2002). Protecting caves is essential for maintaining their unique biodiversity, as a large number of species are highly specialized to live only underground (Moldovan et al. 2018).

Bats are an important part of cave biodiversity and inseparable components of the cave biota. Their guano provides the main source of energy and nutrient for other cavernicolous organisms. Additionally, settling various kinds of parasites, bats in fact, carry a considerable proportion of cave biodiversity with them. Changes in bat-cave relation can accurately reflect the changes in the cave ecosystem; so, bats are used as a reliable criterion in evaluating the degree of alteration and vulnerability of caves. However, bats are exposed to many threats. Cultural attitudes and social unawareness are the main reasons why bat protection is often overlooked, particularly in Iran. Bats have the reputation of being undesirable animals in Iran. Due to plenty of pesticides use in agriculture, bat populations decrease (Karami et al. 2016). According to the results of a recent study, 24 species out of 51 bat species known from Iran are regionally vulnerable, near threatened or data deficient (Yusefi et al. 2019). Although at the global scale, only 13 out of 51 species were classified as vulnerable, near threatened, data deficient, or not evaluated (Yusefi et al. 2019). The results clearly show that we need an extra effort toward bat conservation in Iran.

A prerequisite for any cave conservation program is to have accurate data about its fauna, along with different natural characteristics including location, surface vegetation, water sources, accessibility, etc., and anthropogenic threats such as unsustainable tourism, vandalism, and various human uses. Additionally, to develop strategies for the conservation of caves and their biota, caves should be primarily prioritized using applicable indices. To design an effective and easy-to-apply index to assess cave vulnerability and importance, both biotic and abiotic affinities of caves should be considered.

Furman and Ozgul (2002) designed a simple grading system to evaluate the conservation status of caves as important roosts for bats. This scheme was developed based on bat species abundance data and their conservation status. Tanalgo et al. (2018) introduced a more comprehensive assessment that included different affinities of bat species along with cave geophysical features and human disturbance. They developed the Bat Cave Vulnerability Index (BCVI) which has been used by some other authors in their studies (Deleva and Chaverri 2018).

Since 1865, several researchers have studied the mammalian fauna of Iran including bats (De Filippi 1865; Lay 1967; Farhang Azad 1969; Karami et al. 2008, 2016; Yusefi et al. 2019). In his review of bat research in Iran until the late 1970s, DeBlase (1980) reported 375 records of 38 bat species from Iran along with useful comments on their biology and taxonomy. Benda et al. (2012) presented the most comprehensive

list of bat fauna from Iran. They documented 902 records of 50 bat species in addition to new findings of various aspects of these flying mammals' life. Furthermore, Naderi et al. (2017) added one species to the list of the bat fauna of the country, increasing the number of documented species to 51 including 20 genera from 9 families. In recent years, Iranian researchers have conducted several studies in different parts of the country and provided invaluable information about various aspects of bats' biology in Iran (Akmali et al. 2011a, 2011b, 2015; Fathipour et al. 2016; Shahabi et al. 2017a, 2017b, 2019; Akmali et al. 2019a; Mehdizadeh et al. 2019, 2020; Najafi et al. 2019a, 2019b, 2019c; Akmali 2020; Kafaei et al. 2020a, 2020b). Additionally, Yusefi et al. (2019) provided a comprehensive review of all literature published on terrestrial mammals of Iran including bats along with their latest taxonomic revisions and updated distribution ranges. Several studies on bats of Iran provided some scattered information about the chiropteran fauna of Kurdistan caves (DeBlase 1980; Hemmati 2001; Benda et al. 2012). In these surveys, the occurrence of *Rhinolophus ferrumequinum*, *R. mehelyi*, *R. euryale*, *Myotis blythii*, and *Miniopterus pallidus* was documented. This is the first study that focuses on cave-dwelling bat species from Kurdistan province. It also ranks the caves of the region using BCVI for future conservation management.

The objectives of this study are collecting information about cave-dwelling bats in Kurdistan province in order to upgrade faunal information of the country, and also to be used in future studies including conservation programs. Furthermore, we tried to use a proofed index in order to prioritize Kurdistan caves for future conservation management.

Materials and methods

Description of the Kurdistan province

Kurdistan is located in the west of Iran between 34°44'N to 36°30'N and 45°31'E to 48°16'E, and shares about 200 km of its western political border with Iraq. It neighbors Western Azerbaijan at the north, Hamadan and Zanjan at the east, and Kermanshah province at the south. The altitude range of the region varies from 900 meters in Alout region of Baneh to 3300 meters in Shahou Mountain. Kurdistan can be separated into eastern and western parts which differ in geology and topography, as well as climatic conditions. The eastern part (61% percent of the province area) has a cold and arid climate, while in the western part (39% of the province area), warm and humid climate is dominant. Here, the annual average precipitation is higher than in the eastern part. The western part also includes more forests and rangelands (Gholizadeh and Zarei 2014).

The list of caves in the province was compiled using several information sources. Cooperation was requested from the Environmental Protection Service, the Cultural Heritage and Tourism Organization, as well as other organizations such as caving and mountaineering clubs. All available literature and websites were also surveyed. A total of 28 caves were listed and scheduled for visits.

Field work and data on bat distribution

This study was carried out from 2015 to 2017. To collect information on cave-dwelling bat species in Kurdistan province, several expedition trips were taken in the region. We used direct count method for the caves with a few individuals; and photograph count and population estimation for large colonies. In each cave, a few individuals were captured with the aid of hand-nets and identified according to morphological characteristics using identification keys (DeBlase 1980; Dietz and Von Helversen 2004), then photographed, and released at the same location. The geographical position and altitude of each cave were recorded by Garmin GPS unit (GPSMAP 60CSx; Garmin International, Inc., Olathe, Kansas, USA).

The distribution map of each bat species was drawn by ArcGIS software version 10.2 (ESRI, 2013), using the coordinates obtained during the field work.

Assessing cave conservation vulnerability

Data on cave geophysical characteristics and human activities were obtained from our direct observation during field works. Accessibility to the cave sites, cave openings, and effort of exploration in the caves were determined by direct observation, experience, and comparison of different caves. Data about tourism activity, cave use, and Land-use change activities within cave vicinity were gathered from direct observation, local people and, tourism companies. All the information was listed; each parameter was separately scored for each cave in comparison with other caves.

We used the Bat Cave Vulnerability Index (**BCVI**) proposed by Tanalgo et al. (2018) to measure the conservation priorities of Kurdistan caves. The index has two components including Biotic Potential (**BP**) and Biotic Vulnerability (**BV**) which are separately calculated. The final value which is represented as an alphanumeric index is a combination of the scores from both components for each cave. BP and BV are calculated in different ways. Based on our data sources, we calculated the value of each component and then scored them according to Tanalgo et al. (2018).

BP value for each cave is the sum of the computed scores for individual bat species, multiplied by total species richness. The numeric values of abundance, relative abundance, endemism, and conservation status, and species-site commonness of bat species are the basis of this assessment

Species richness is the number of bat species per cave, using information from our field work.

Species abundance is the number of individuals of each species per cave.

Species relative abundance was calculated using the number of individuals of each species in a specific cave dividing by average abundance of that species from all caves where the species was observed.

Species-site commonness is a value for determining the rarity of species. This parameter is calculated using the formula below:

"The number of all caves assessed in the study/ frequency of the species occurrence", where the frequency means the number of caves that a specific species was observed.

Determining the endemism and conservation status of each species were done by using the latest information from the International Union for the Conservation of Nature (IUCN) Red List (www.iucnredlist.org). Each category then was translated to a score according to the scoring system as in Tanalgo et al. (2018). In this system, widespread, regional endemism, country endemism and restricted or data deficient are given 2, 3, 4 and 5 scores, respectively. Also, least concern, least concern with decreasing population, near threatened, vulnerable, and endangered are given 2, 3, 4 and 5 scores, respectively. Score 6 goes to critically endangered, near extinction, and data deficient species (Tanalgo et al. 2018). After listing all the scores, we applied the formula proposed by Tanalgo et al. (2018).

BV value is obtained by scoring the geophysical and anthropogenic status of the caves. Geophysical and anthropogenic characteristics include six parameters as shown in Table 5. Accessibility to cave sites, cave openings, the effort of exploration, tourism activity, cave use, and Land-use change activities within cave vicinity are the six parameters that are considered in the study.

Calculating BCVI values

The next step is to combine BP and BV indices to construct BCVI values. As shown in Fig. 1 adopted from Tanalgo et al. (2018), 16 possible ranks are derived by combining the BP and BV values, which are categorized under three levels of priority: high, moderate, and low.

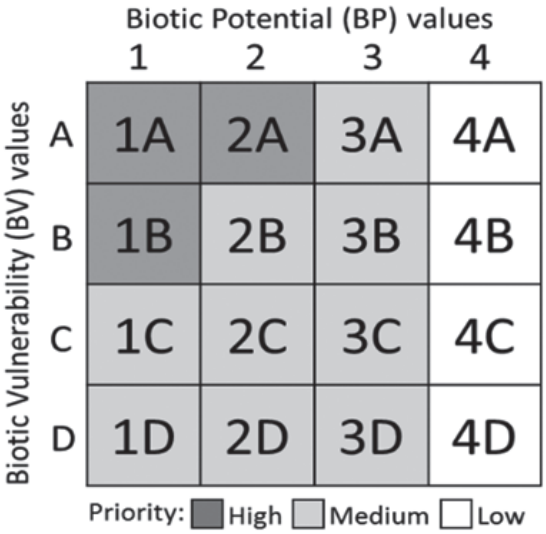

Figure 1. 16 possible alphanumerical BCVI values resulted from a combination of BP and BV scores. Cave priorities are categorized into three groups: High priority, medium priority, and Low priority.

Results

New bat records from inventories in 2015–2017

From 28 explored caves, all but two caves (Div Zendan and Kani Gureh) were occupied with at least one bat species (Tables 1; Suppl. material 1: Fig. S1). Six caves with one species, ten caves with two, six caves harboring three species, two caves with four species, as well as one cave with five and another one with six species were recorded (Table 2).

Overall, 10 bat species belonging to four families were documented in the region under study (Tables 1, 2). Furthermore, in two caves including Davazdah Emam and Golestaneh, unidentified rhinolophid bats were observed that we refer them to *Rhinolophus* sp. (Tables 1, 2).

Table 1. The list of 28 caves investigated in Kurdistan Province, with closest town and geographic coordinates of the cave entrances. If a bat was recorded, a plus sign is given. Species names are coded with numbers: 1 – *Rhinolophus ferrumequinum*, 2 – *R. mehelyi*, 3 – *R. euryale*, 4 – *R. blasii*, 5 – *R. hipposideros*, 6 – *Myotis blythii*, 7 – *M. emarginatus*, 8 – *Plecotus macrobullaris*, 9 – *Miniopterus pallidus*, 10 – *Tadarida teniotis*, 11 – *Rhinolophus* sp. The total number of species is given in the last column.

No.	Cave name	Town	Coordinates	1	2	3	4	5	6	7	8	9	10	11	Total	Date of observation
1	Sayel Mayel	Bijar	36°00'27"N, 47°35'03"E	+	+	+				+					4	7,7,2016
2	Salavat Abad	Bijar	36°00'58"N, 47°34'53"E	+											1	10,5,2016
3	Soltan Abad	Bijar	36°19'27"N, 47°26'47"E						+			+			2	21,7,2016
4	Zaqeh Rash	Bijar	36°10'15"N, 47°36'47"E						+			+			2	22,7,2016
5	Zaqeh Tarik	Bijar	35°47'23"N, 47°29'24"E			+			+			+			3	17,5,2017
6	Davazdah Emam	Bijar	35°48'20"N, 47°26'48"E									+		+	2	17,5,2017
7	KaniRahman Qiseh	Bijar	35°55'15"N, 47°28'51"E	+							+				2	26,5,2017
8	Gar Emam	Bijar	35°57'53"N, 47°33'23"E									+			1	23,7,2016
9	Kamtaran	Bijar	36°00'12" N, 47°34'54"E	+	+	+	+		+			+			6	2,8,2016
10	Garchang Siyah	Bijar	36°00'31"N, 47°32'44"E										+		1	5,9,2016
11	Seyyed Bolqaa	Bijar	36°01'01"N, 47°40'03"E	+					+	+		+			4	20,5,2017
12	Posht-e Darband	Bijar	36°00'39"N, 47°35'39"E	+	+										2	25,5,2017
13	Nesar	Bijar	35°52'26"N, 47°33'49"E									+			1	26,5,2017
14	Golestaneh	Bijar	35°54'53"N, 47°54'53"E									+		+	2	14,6,2015
15	Div Zendan	Qorveh	35°07'52"N, 47°42'34"E												0	16,11,2016
16	Farhad Tash	Qorveh	35°01'17"N, 47°08'00"E						+			+			2	23,5,2017
17	Charmeh Savar	Qorveh	35°33'08"N, 47°48'13"E	+					+			+			3	24,5,2017
18	Mohammad Saleh	Qorveh	35°33'45"N, 47°47'07"E	+											1	24,5,2017
19	Zaqeh Guri	Qorveh	35°07'44"N, 47°35'41"E	+					+						2	23,5,2017
20	Kulava	Qorveh	35°09'23"N, 47°46'22"E						+			+			2	27,5,2017
21	Kani Gureh	Dehgolan	35°26'12"N, 47°33'01"E												0	15,11,2016
22	Samangan	Divan Dareh	36°07'47"N, 47°03'11"E	+											1	12,6,2015
23	Tarik	Divan Dareh	36°07'01"N, 47°05'07"E		+						+	+			3	13,6,2015
24	Tarik 2	Divan Dareh	36°07'06"N, 47°05'15"E		+							+			2	13,6,2015
25	Karaftu	Divan Dareh	36°19'08"N, 46°52'06"E	+	+			+	+			+			5	16,6,2015
26	Shouvi	Baneh	36°00'18"N, 45°53'45"E			+	+		+						3	16,6,2015
27	Darvish Ouliya	Marivan	35°37'14"N, 46°37'59"E		+		+		+						3	10,5,2016
28	Kouna Sham- Sham	Sanandaj	35°41'10"N, 46°26'36"E	+					+			+			3	11,5,2016

Table 2. The number of caves for each species, as well as their altitudinal ranges and observation time.

No.	Species	Number of caves	Altitudinal range (m)	Observation time
1	*Rhinolophus ferrumequinum*	12	1621–2249	Spring and summer
2	*R. mehelyi*	7	1621–2160	Spring and summer
3	*R. euryale*	4	1642–2013	Spring and summer
4	*R. blasii*	3	1804–2160	Spring and summer
5	*R. hipposideros*	1	2000	Spring
6	*Myotis blythii*	13	1626–2263	Spring and summer
7	*M. emarginatus*	2	1626–1642	Summer and autumn
8	*Plecotus macrobullaris*	2	1915–2176	Spring and summer
9	*Miniopterus pallidus*	16	1626–2348	Spring and summer
10	*Tadarida teniotis*	1	1666	Summer
11	*Rhinolophus* sp.	2	1810–1959	Spring and summer

Table 3. Composition of the bat fauna of Kurdistan province and the number of records for each species based on Benda et al. (2012) compared with the new records from the current study.

Family	Species	Benda et al. (2012)	The current study	Total records for Kurdistan	Total records for Iran based on Yusefi et al. (2019) and Akmali et al. (2019a)
Rhinolophidae	*Rhinolophus ferrumequinum*	2	12	14	111
	Rhinolophus hipposideros	0	1	1	55
	Rhinolophus euryale	2	4	6	28
	Rhinolophus mehelyi	4	7	11	18
	Rhinolophus blasii	0	3	3	38
Vespertilionidae	*Myotis blythii*	9	13	22	150
	Myotis emarginatus	0	2	2	23
	Plecotus macrobullaris	0	2	2	14
Molossidae	*Tadarida teniotis*	0	1	1	26
Miniopteridae	*Miniopterus pallidus*	8	16	24	76
Total		25	61	86	539

The most widespread species was *Miniopterus pallidus* which was observed in 16 caves (Tables 1, 2; Suppl. material 1: Figs S1, S2). *R. hipposideros* and *T. teniotis* have the lowest distribution ranges, each occurring only in one cave. The largest colonies belong to *M. pallidus* and *Myotis blythii*. Besides, *T. teniotis*, and *P. macrobullaris* have the smallest population size with two individuals observed for each species

In Table 3, we give overview of records for each species for Kurdistan region before our study, and information on increase in number of records, as result of our investigations.

Identifying priority caves for bat conservation

The results from the calculations of BCVI for 26 caves are shown in Table 5. Population size and species richness are also given for each cave. Information about endemicity and conservation status of each species and their scoring are presented in Table 4.

Table 5 summarizes all the scores and calculated BP, BV scores and the resulting BCVI index values.

Table 4. Endemism and conservation status and scores for each species observed in Kurdistan caves. Score number follows the methodology in Tanalgo et al. 2018.

No.	Species	Endemism	Score	Conservation status	Score
1	*Rhinolophus ferrumequinum*	Regional endemic	3	Least concern with decreasing population	3
2	*Rhinolophus hipposideros*	Regional endemic	3	Least concern with decreasing population	3
3	*Rhinolophus euryale*	Regional endemic	3	Near threatened	3
4	*Rhinolophus mehelyi*	Regional endemic	3	Vulnerable	4
5	*Rhinolophus blasii*	Regional endemic	3	Least concern with decreasing population	3
6	*Myotis blythii*	Regional endemic	3	Least concern with decreasing population	3
7	*Myotis emarginatus*	Regional endemic	3	Least concern	2
8	*Plecotus macrobullaris*	Regional endemic	3	Least concern with decreasing population	3
9	*Tadarida teniotis*	Regional endemic	3	Least concern	2
10	*Miniopterus pallidus*	Regional endemic	3	Near threatened	3

Table 5. BCVI values and total BCVI scores for Kurdistan caves. Geophysical and human activity features are classified as below: 1 – Accessibility to cave sites, 2 – Cave openings, 3 – Effort of exploration, 4 – Tourism Activity, 5 – Cave use, 6 – Land-use change activities within cave vicinity. Scores follow the methods in Tanaglo et al. 2018.

No.	cave	Estimated population	Species richness	BP Score	BP Index	Geophysical and human activity features						BV Score	BV Index	Total BCVI Value
						1	2	3	4	5	6			
1	Sayel Mayel	134	4	18787.60	4	2	1	2	2	4	2	2.16	B	4B
2	Salavat Abad	20	1	134.48	4	2	1	2	4	4	3	2.66	B	4B
3	Soltan Abad	500	2	23742.72	3	2	2	2	3	4	3	2.66	B	3B
4	Zaqeh Rash	30	2	111.11	4	2	1	2	3	4	3	2.5	B	4B
5	Zaqeh Tarik	20	3	170.19	4	2	2	2	4	4	2	2.66	B	4B
6	Davazdah Emam	2	2	0.49	4	2	2	2	4	4	2	2.66	B	4B
7	Kani Rahman Qiseh	7	2	16.54	4	2	1	1	4	4	2	2.33	B	4B
8	Gar Emam	1	1	0.11	4	2	2	2	4	4	2	2.66	B	4B
9	Kamtaran	850	6	229986.90	1	2	1	2	2	3	3	2.16	B	1B
10	Garchang Siyah	2	1	348	4	1	2	1	3	4	2	2.16	B	4B
11	Seyyed Bolqaa	500	4	70054.60	2	2	3	3	4	4	3	3.16	C	2B
12	Posht-e Darband	5	2	11.47	4	2	2	2	4	4	3	2.83	B	4B
13	Nesar	2	1	0.48	4	1	1	2	3	4	3	2.33	B	4B
14	Golestaneh	101	2	1254.30	4	2	3	3	3	4	3	3	C	4B
15	Farhad Tash	30	2	111.10	4	2	3	2	4	4	3	3	C	4C
16	Charmeh Savar	30	3	140.90	4	2	1	1	4	4	3	2.5	B	4B
17	Mohammad Saleh	3	1	3.05	4	2	1	1	4	4	3	2.5	B	4B
18	Zaqeh Guri	30	2	201.90	4	2	1	2	4	4	3	2.66	B	4B
19	Kulava	15	2	28.68	4	1	2	2	4	4	3	2.66	B	4B
20	Samangan	2	1	1.34	4	2	3	2	4	4	3	3	C	4C
21	Tarik	111	3	4603.93	4	2	1	1	4	3	4	2.5	B	4B
22	Tarik 2	470	2	57741.39	3	2	1	1	4	4	4	2.66	B	3B
23	Karaftu	1150	5	427904.73	1	2	2	2	1	4	4	2.5	B	1B
24	Shouvi	185	3	10199.94	4	2	2	2	4	4	2	2.66	B	4B
25	Darvish Ouliya	770	3	187896.72	1	2	2	2	3	3	3	2.5	B	1B
26	Kouna Sham-Sham	1200	3	144332.28	1	2	1	2	2	3	3	2.16	B	1B

Four caves including Kamtaran, Karaftu, Darvish Ouliya, and Kouna Sham-Sham (Fig. 2) received the highest priority getting a 1B value. All these caves were the most populous in this study (with 770–1200 estimated individuals). Species richness of

these four caves varies from 3 to 6. All of them are easily accessible and the entrances are rather easy to pass with minimum obstacles inside them. Three caves including Soltan Abad and Tarik2 (3B), and Seyyed Bolqaa (2C) are moderately vulnerable. In these three caves, no or minimum sign of cave use, tourism, and land-use change activities were observed. About 500 individuals were counted in each of these three caves at the time of the investigation. The remaining 19 caves get 4B or 4C values which are commented to have the lowest priority. Although bat diversity of these 19 caves ranges from one to four species, only 30 or fewer individuals were counted in 15 of them. It is concluded that population size is the most effective parameter in computing the final BCVI value for studied caves in Kurdistan province. For example, both Sayel Mayel and Seyyed Bolqaa caves harbor four bat species, but the former with 134 individuals is classified as low, and the latter with 500 shows the moderate priority. Additionally, Shouvi with 185 and Kouna Sham-Sham with 1200 individuals get low and high priority index, respectively. Both are the same in term of their bat diversity.

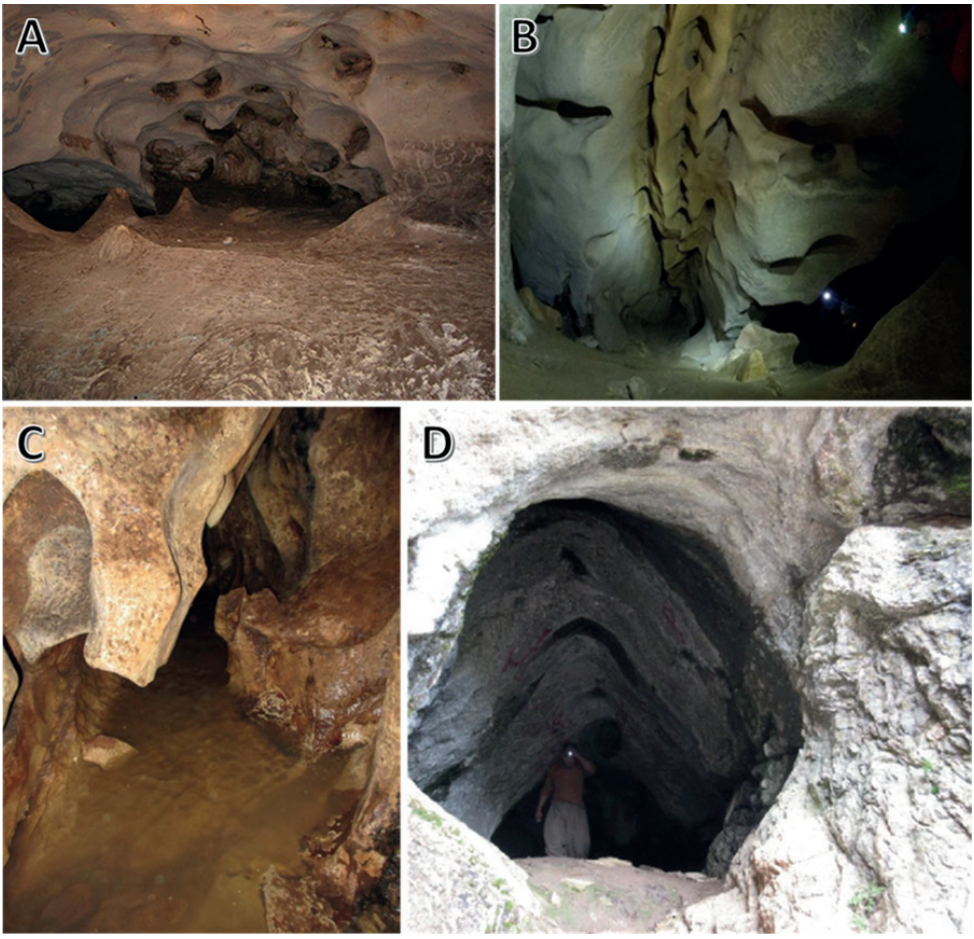

Figure 2. Four caves of Kurdistan with the highest priority for conservation programs: **A** Karaftu **B** Kamtaran **C** Darvish Ouliya **D** Kouna Sham-Sham.

Discussion

Improved knowledge on bat species in the Kurdistan region

Our study increased the number of bat species in Kurdistan to 10. This study demonstrates that the species richness of the region is much higher than previously assumed, and it raises the respective number to 10 species. Out of this number, three species are globally in decline.

So far, up to 1311 bat records have been documented in Iran (Akmali et al. 2019a; Yusefi et al. 2019), from which 25 records refer to Kurdistan province (Benda et al. 2012). The results of the current study upgrade the respective number of Iran to 1372 records. Also, the records of Kurdistan reach 86 which shows the province covers approximately 16 percent of the whole country records on bats.

Only five bat species were recorded in previous studies from Kurdistan province.

Overview of species with comments on distribution and conservation

The identified species represent five genera from four Families and are listed as below:

Family Rhinolophidae Gray, 1825

Rhinolophus ferrumequinum (Schreber, 1774)

The greater horseshoe bat occurs in 12 caves. The abundance of this bat ranges from two to many individuals in mixed colonies. Previously, Benda et al. (2012) had reported this species from Karaftu cave in Divan Dareh. Akmali et al. (2011b) have also observed this species in Karaftu cave.

Rhinolophus mehelyi Matschie, 1901

The Mehely's horseshoe bat, was observed in seven caves. It occurs in different numbers; from a few individuals to small or big colonies mixed with other species. There are several previous reports on the occurrence of this species in Kurdistan caves. In August 1968, DeBlase (1980) reported a huge colony of 30000 individuals mix of *R. mehelyi*, *Myotis blythii* and *Miniopterus pallidus* in Tarik cave, from which 12000 were estimated to be *R. mehelyi*. Tarik cave is one of the caves that was studied in the current work, but a noticeable decline in its bat population was obvious. Furthermore, Hemmati (2001) has recorded a nursery colony in Aftabi cave, one male in zivieh cave, and three male individuals in Karaftu cave.

Rhinolophus euryale Blasius, 1853

The Mediterranean horseshoe bat occurs widely throughout the Zagros Mountain ranges. Previously, DeBlase (1980) had reported this bat from a religious shrine near

Bijar. Additionally, Benda et al. (2012) observed one female individual in Sanandaj, west of Kurdistan. In the current study, *R. euryale* was observed in more caves, where it co-occurs with other species.

Rhinolophus hipposideros (Borkhausen, 1797)

This is the first report on the occurrence of the lesser horseshoe bat in Kurdistan. According to the literature, this species occurs in three adjacent provinces of Kurdistan including Azarbaijan-e Gharbi, Kermanshah and Zanjan (Benda et al. 2012).

Rhinolophus blasii Peters, 1866

There was no previous record of Blasius's horseshoe bat in Kurdistan province (Benda et al. 2012). Although *R. blasii* is one of the most widespread bat species in Iran, it is not widely distributed in the westernmost parts of the country. In this study, *R. blasii* was identified in large colonies co-occurring with other species.

Family Vespertilionidae Gray, 1821

Myotis blythii (Tomes, 1857)

The lesser mouse-eared bat is a very common bat in Iran. It is widely distributed across the mountainous parts of the country in the north, west and south-west (Benda et al. 2012). DeBlase (1980) has documented the observation of three males in a cave north-east of Bijar; and 30 individuals in Karaftu cave, Divan Dareh. Furthermore, Hemmati (2001) has collected nine males from this cave. Observation of three individuals was also reported from Karaftu cave (Benda et al. 2012). From the largest aggregation of *M. blythii* in Iran including 12000 individuals, DeBlase (1980) collected and examined about 300 in Tarik (Gara Tarik) cave, Divan Dareh. Also, Hemmati (2001) visited this cave and collected three males and four females of this species. The same author has recorded four males and five females from a possible nursery roost in Zivieh cave, 40 km away from east of Saqqez in the north-west of the province. Finally, DeBlase (1980) has reported 20 *M. blythii* specimens from a mosque dome in Sanandaj, west of the province.

In this study, *M. blythii* was recorded from 13 caves. Simultaneous occurrence of this species and *Miniopterus pallidus* is evident in 10 out 13 caves in which mixed colonies comprises 10 to hundreds of individuals. There is no cave where the *M. blythii* species occurs solely.

Myotis emarginatus (Geoffroy, 1806)

This is the first report of Geoffroy's bat from Kurdistan province. Previous records from Iran shows that, this species occurs in very different climatic zones, from Caspian

coastal plain to south-easternmost region of the east-Baluchestani mountain ranges. Akmali et al. (2011b) and DeBlase (1980) have reported this species from Kermanshah province, the western neighbor of Kurdistan.

Plecotus macrobullaris Kuzâkin, 1965

This is the first report on the occurrence of Alpine Long-eared bat from Kurdistan; although, it was recorded in three adjacent provinces including Azabaijan-e Gharbi, Zanjan and Hamadan (DeBlase 1980; Benda et al. 2004; Juste et al. 2004; Spitzenberger et al. 2006; Hemmati 2009). Totally, the records on this species are confined to western half of the country. In this study, the observation of two individuals of *P. macrobullaris* from two separate caves was documented.

Family Miniopteridae Dobson, 1875

Miniopterus pallidus Thomas, 1907

Pale Bent-winged Bat is a common bat species in Iran. There are several records on the occurrence of this species in Kurdistan province. DeBlase (1980) has observed a colony of 5500 individuals in Tarik cave. The same author has also reported 25 individuals from Karaftu cave, Divan Dareh. Hemmati (2001) visited this cave and reported 12 male individuals of M. pallidus. Furthermore, Karatas et al. (2008) and Furman et al. (2009, 2010), each collected two individuals from their roost in Karaftu cave. Also, Benda et al. (2012) have reported on the observation of 100 individuals in torpor from the same cave. It's confirmed that Karaftu cave is used by *M. pallidus* as a roost year-round; and this bat has occurred continually over long periods in this cave. In their study, Akmali et al. (2011b) have documented the occurrence of this species in Zivieh cave, Saqqez. One female individual was also collected from Aftabi cave (Hemmati 2001).

In the current study, *M. pallidus* was found to be the most widespread cave-dwelling species throughout the east Kurdistan, occurring in 16 caves. Its population size differs from a few individuals to large colonies, either solely or mixed with other species.

Family Molossidae

Tadarida teniotis (Rafinesque, 1814)

European Free-tailed bat, *Tadarida teniotis*, is recorded for the first time from Kurdistan. Two individuals were collected from one cave. Excluding the record of echolocation calls from five foraging individuals in Kermanshah (Benda et al. 2012), there is no previous report on the occurrence of this species in Kurdistan and its neighbor provinces.

Rhinolophus mehelyi is categorized as vulnerable (VU) both regionally and globally. Compared with previous reports on this species, a noticeable decline in the population of *R. mehelyi* is obvious in the region under study.

Miniopterus pallidus is the other species that is globally known as near threatened (NT). In the country, this species is classified as VU. In the current study, *M. pallidus* showed a wide distribution range occurring in 16 species and an altitudinal preference from 1626 to 2348 m.

Such extensive occurrence ranges proofs that the caves of Iran, especially those in the Kurdistan region can be utilized as long-term colonization sites and permanent roosts by different bat species. Nevertheless, effective conservation strategies should be adopted to protect them and their biodiversity. A good example is Tarik Cave where DeBlase (1980) observed several thousand bats in a mixed colony of *Rhinolophus mehelyi*, *Myotis blythii*, and *Miniopterus pallidus.*

Rhinolophus euryale is another species that is ranked as NT according to the global IUCN. However, this species is classified in the Least Concern (LC) category in Iran. Reversely, *Rhinplophus hipposideros* and *R. ferrumequinum* which are known as VU in Iran are globally categorized as LC.

Such data can be used to prioritize bat species and consequently, their roost sites in conservation management. This study showed that four species including *Rhinolophus mehelyi*, *R. hipposideros*, *R. ferrumequinum*, and *Miniopterus pallidus* should be considered as a priority. Consequently, the caves that harbor the majority of these threatened species should receive particular attention in any conservation effort. To support this assumption, we conducted a prioritization analysis using the Bat Cave Vulnerability Index (BCVI).

Prioritization of caves according to BCVI values

Only five from 16 possible BCVI values (Tanalgo et al 2018) were obtained for Kurdistan caves. It shows that, regardless high variation in population size (from 1 to 1200 individuals), and diversity (1 to 6 species) per cave, BCVI couldn't adequately categorize Kurdistan caves and more specified parameters are essential for a particular region. In a holistic assessment, all BV parameters get the same weight; so, two caves with similar BV scores do not necessarily have equal conservation importance. As developers of BCVI commented, the parameters should be modified in particular conservation programs with different purposes (Tanalgo et al. 2018). This means that we may manipulate the BV criteria according to our conservation strategy.

Another point is, as some species have different global and regional IUCN statuses (Yusefi et al. 2019), the regional conservation status should also be considered in our assessment if we want to have more accurate results for a particular country.

Karaftu cave is the only cave whose bat fauna contains all four species and this is considered a priority in this study. This cave is one of the longest caves in the region and is divided into two parts: the artificial part and the natural part. A permanent water source exists in the cave and many passages are branched from this four-floored cave. This cave was one of the main summer roosts for bat species in the past (DeBlase 1980). With a bat fauna including five species, this cave deserves to be treated as a hotspot for bats. Another cave that has the highest species richness in the province is Kamtaran

Cave. Besides, some fossil remnants which resemble prehistoric creatures were observed on the floor and roof of this cave. It is one of the main roosts of bats and should be considered a hot spot for them and receive immediate conservational attention. Darvish Ouliya is one of the main roosts for *R. blasii* which is considered a rare species over the region. A water stream is running on the floor finding its way outside the cave. The largest mixed colony is observed in Kouna Sham-Sham with 1200 individuals. This cave is also one of the largest caves with three entrances and numerous inner branches.

Conclusion

This study improved the information on cave dwelling bats and their roosts in Kurdistan province. Although BCVI is a relatively efficient index to prioritize caves, some modifications can be included. Regional IUCN categorization may differ from global IUCN; therefore, it is important to consider it in our assessments. Another important issue is the type of roost in each cave. It is better to score caves according to the particular use by bats. For example, a cave with nursery colonies can be scored higher than a cave with transient roosts. This index could not effectively categorize the caves with low conservation priorities; therefore, we think more than four levels of grading for BC and BV values are needed. Seasonal monitoring of bat populations is also important, since the population size is the most determining parameter in this index. Finally, we suggest designing a more comprehensive index for the caves of Iran in future studies.

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Supplementary material 1

Figures S1, S2

Authors: Vahid Akmali, Siavash Abedini, Zahra Malekpour Fard

Data type: images

Explanation note: Figure S1. The geographical location of Kurdistan Province in the west of Iran and 28 caves investigated in the study area. Numbers refer to caves as indicated in Table 1. Figure S2. Separate distribution maps for each bat species with their site numbers.

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RESEARCH ARTICLE

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A new obligate groundwater species of *Asellus* (Isopoda, Asellidae) from Iran

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Abstract

With only 43 described stygobionts and only two isopod species the obligate groundwater fauna of Iran, a vast country with over 10% of limestone surface, is inadequately known. Here, we report the discovery of *Asellus ismailsezarii* sp. nov. from Zagros mountains, the first eyeless and depigmented asellid isopod from Iran. The new species is morphologically similar to *Asellus monticola* Birstein, 1932, but it is eyeless and fully depigmented, has a slightly curved pereopod IV and does not bear any setae on proximal margins of exopodite of pleopods IV and V. Species phylogenetic relationships using original and datamined mitochondrial DNA and nuclear rDNA, and estimation of molecular divergences with other *Asellus* species, suggest that *A. ismailsezarii* sp. nov. is sister to a larger clade that also contains the European *A. aquaticus* species complex. Surface populations of *Asellus* have colonized groundwater at multiple occasions and localities, both in Europe and Asia, giving rise to species and subspecies that have evolved troglomorphisms, such as depigmentation and loss of eyes. Of the 37 formally described species and subspecies of *Asellus*, 15 are from groundwater, including *A. ismailsezarii* sp. nov. We predict that many more obligate groundwater *Asellus* taxa are yet to be discovered in Asia.

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Keywords
Asia, Crustacea, groundwater, molecular phylogeny, taxonomy, troglomorphy

Introduction

Groundwater harbors a high diversity of metazoans that represents an important, yet underestimated, component of the Earth's freshwater biodiversity (Culver and Holsinger 1992; Dole-Olivier et al. 2009). Knowledge of groundwater biodiversity is uneven across the globe: species inventories are far more advanced in Europe, North America, and Australia, for example, than in South America, Africa and Asia (Zagmajster et al. 2018). We know of only 43 obligate groundwater species from Iran, despite the fact that 10.5% of the country (compared with approximately 1.7% of Europe) is underlain by carbonate rock formations. The obligate groundwater fauna includes four fish species, 23 amphipods of the genus *Niphargus* Schiødte, 1847, 12 copepods, one oligochaete, one gastropod and two isopods (Cichocka et al. 2015; Mousavi-Sabet et al. 2016; Malek-Hosseini and Zamani 2017; Vatandoust et al. 2019; Bargrizaneh et al. 2021; Malek-Hosseini et al. 2021). The only two obligate groundwater isopods known from Iran are *Microcharon raffaellae* Pesce, 1979 (Lepidocharontidae) collected from a well in Pliocenic sandstone and clay at Shahr-e-Kord city, Chaharmahal and Bakhtiari Province, and *Stenasellus tashanicus* Khalaji-Pirbalouty, Fatemi, Malek-Hosseini & Kuntner, 2018 (Stenasellidae) collected from Tashan karstic cave in Khuzestan province (Pesce 1979; Khalaji-Pirbalouty et al. 2018). In the present article, we describe the third obligate groundwater isopod from Iran belonging to the family Asellidae (Pancrustacea, Isopoda, Asellota).

The Asellidae is one of the few families of metazoans containing a large number of both surface and subterranean aquatic species. To date, the family contains 23 genera and 428 species and subspecies (Malard et al. in press). Of these 428 species and subspecies, 279 are eyeless and depigmented obligate groundwater species, 18 are mostly confined to groundwater, often showing reduced eyes and pigmentation, and 131 are occulated and pigmented surface water species. The family is widely distributed in the northern hemisphere with species in northern part of North America, Europe, northern Africa and Asia. The systematics of asellids is not yet fully established but morphological and molecular evidence support the existence of three major clades whose species occur in America, Europe and Northern Africa, and Asia, respectively (Morvan et al. 2013). One of these major clades to which Henry and Magniez (1995) referred to as the "*Asellus* pattern" contains 59 species and subspecies among which 26 inhabit surface waters and 33 groundwaters. They belong to the genera *Asellus* Geoffroy, 1762, *Calasellus* Bowman, 1981, *Columbasellus* Lewis, Martin & Wetzer, 2003, *Limnoasellus* (*Nomen nudum* in Hidding et al. 2003), *Mesoasellus* Birstein, 1939, *Nipponasellus* Matsumoto, 1962, *Phreatoasellus* Matsumoto, 1962, *Sibirasellus* Henry & Magniez, 1993 and *Uenasellus* Matsumoto, 1962.

With the exception of the genus *Asellus*, all genera within the "*Asellus* pattern" have relatively narrow distribution ranges either in Lake Baikal, Far East Russia, South Korea, Japan, or the Pacific Northwest coast of North America (Matsumoto 1963, 1966;

Bowman 1981; Henry and Magniez 1991, 1993, 1995; Hidding et al. 2003; Lewis et al. 2003; Sidorov and Prevorčnik 2016). The genus *Asellus* shows a wide geographic range, being present in Alaska (1 species, Bowman and Holmquist 1975), Asia (15 species, Henry and Magniez 1995; Sidorov and Prevorčnik 2016) and Europe (2 species). The occurrences of *Asellus* in Europe were all attributed to the *Asellus aquaticus sensu lato* species complex (Verovnik et al. 2005), with the exception of occurrences from two nearby surface water sites in eastern Ukraine, which were attributed to *Asellus* sp. (River Kharkiv and Pionersky pond; see Verovnik et al. 2005). The taxonomic status of *Asellus* populations within the *A. aquaticus* complex is not yet fully resolved. That complex actually contains two species, *A. aquaticus* Linnaeus, 1758 and *A. kosswigi* Verovnik et al., 2009, and eleven formally described subspecies (Sket 1965; Turk-Prevorčnik and Blejec 1998). COI-based species delimitation methods indicate that there may be many more species (Sworobowicz et al. 2015, 2020). Also, several *Asellus* populations belonging to that complex have colonized caves in Europe and have evolved traits such as loss of pigmentation and eye reduction (Balázs et al. 2021).

The eastern geographic boundaries of the *A. aquaticus* complex are not clearly established. The global biodiversity information facility does not report any records of *A. aquaticus* Linnaeus, 1758 in Iran (consultation date: 16/11/2021). However, the presence of that species was reported by Rémy (1941) at a pond located 6 km from Chahi, Mazanderan province, and more recently by Henry and Magniez (1995) at a surface freshwater site in the Gorgan province contiguous to Turkmenistan. Birstein (1945) described *Asellus aquaticus messerianus* from Turkmenistan Lakes Delili, Yashka and Karatogelek located near the northern border of Iran. Another asellid species, *Asellus monticola*, originally described by Birstein (1932) from freshwater surface sites in Armenia and Georgia was collected in Northern Iran at Bora Laan Spring, a locality located near the Turkish border (Henry and Magniez 1996).

Here, we report on the discovery of *Asellus ismailsezarii* sp. nov. (Asellidae, Asellota, Isopoda, Pancrustacea), the first eyeless and depigmented asellid isopod described from Iran. We also provide morphological comparisons with *A. monticola*, the species that morphologically resembles *Asellus ismailsezarii* sp. nov. as well as comparisons with European cave-species and subspecies belonging to the *A. aquaticus* complex. We then use molecular data to corroborate the species status of *A. ismailsezarii* sp. nov. and to document its phylogenetic relationship to the *A. aquaticus* complex.

Material and methods

Sampling

On several occasions from December 2018 to June 2019, we collected by hand four males, four females and 17 juveniles of *Asellus* at Ganow (Gandab) spring, Iran. This karstic spring is seasonal, flowing during winter and spring. The spring is located close to Tuveh village, Andimeshk, Khuzestan Province (geographic coordinates: 32°48'31"N, 48°43'32"E; altitude: 470 m above sea level) (Fig. 1).

Figure 1. Photo of Ganow spring, the type locality of *Asellus ismailsezarii* sp. nov., Tuveh village, Andimeshk, Khuzestan Province, Iran. Stream width is about one meter.

Morphological description

When necessary, pereopods I, IV and VII from one side of the body were dissected and heat-treated in a KOH solution, dyed with Trypan Blue for better visibility of spinulation and then temporary mounted on slides in glycerine. After dissection, also antennae I, II, pleopods, uropods and pleotelson, were mounted on slides in glycerine. Specimens and their body parts were photographed and measured using a Sony DXC390P digital camera mounted on a stereomicroscope or microscope (depending on the size of the structure), and measured with Leica Application suite – LAS EZ. The remains of the dissected specimens were then transferred to 70% ethanol for storage. In one of the specimens from the type locality, all appendages were dissected and prepared for drawing. All pereopods, as well as the trunk were heat-treated and dyed as described above, and then temporarily mounted in glycerine, alongside the rest of dissected specimen (mouth appendages, antennae, pleopods, uropods). Vector drawings were made from microphotographs using a graphics tablet (Wacom, Cintiq 13HD Creative Pen Display) and the free software KRITA 4.1.1 (https://krita.ord/). In males, we measured 90 morphometric characters (cf. Prevorčnik et al. 2004) for describing body proportions (trunk, appendages) and characterizing cuticular and sensory structures (number and length of spines, setae, aesthetascs). In antenna II, only the last two articles of the

antennal basis (fourth and fifth) were included in the total antennal length together with the length of the flagellum. In pereopods IV and VII, length of the coxa was not included in the total pereopod length. We did not report full length of antenna II and pereopods because in most cases the basal articles of both appendages remained attached to the body during dissection. Throughout the ensuing description, we provide a single measurement value for the holotype, together with a range of values (in parentheses) referring to the males (♂♂) and females (♀♀) from the type material.

For observation with scanning electron microscopy (SEM), Plp I and Plp II of the male holotype and paratype stored in 70% ethanol were air-dried and mounted on sample stubs using conductive double sided carbon tape. Mounted samples were sputter-coated with platinum and observed with a JEOL JSM-7500F field emission scanning electron microscope (Jeol. Japan) at the Department of Biology, Biotechnical Faculty, University of Ljubljana.

Molecular taxonomy

DNA extraction, amplification and gene sequencing

We used the last three pereopods of three specimens (specimens AS1, AS2 and AS3, see Material examined) of *A. ismailsezarii* sp. nov. to obtain sequences of the mitochondrial cytochrome oxidase subunit I (COI) gene and 28S nuclear rDNA gene. We performed molecular analyses at the Evolutionary Zoology Laboratory, Jovan Hadži Institute of Biology ZRC SAZU, Ljubljana (EZ LAB, Slovenia) and LEHNA laboratory, Villeurbanne (LEHNA, France). We crosschecked DNA extraction protocols for the COI gene using specimen AS1 and DNA extraction and PCR protocols for the 28S using specimens AS1 and AS2. Full detail of the molecular protocol for each gene and specimen is provided in Suppl. material 1: Table S1 and Suppl. material 2: Table S2.

We performed robotic DNA extraction at EZ LAB on the three specimens using Mag MAX™ Express magnetic particle processor Type 700 with DNA Multisample kit (Thermo Fisher Scientific kit) and modified protocols following Vidergar et al. (2014). We also extracted DNA from specimen AS1 at LEHNA using a modified Chelex protocol from Casquet et al. (2012). We incubated pereopods in a solution of 150 µl of 7% chelex and 10 µl of proteinase K at 15 mg / ml for 90 minutes at 56 °C, then 15 minutes at 90 °C.

We performed polymerase chain reactions (PCRs) for COI fragments at LEHNA using a previously optimized protocol (Calvignac et al. 2011). However, we used a Taq polymerase (Eurobiotaq) amount of 0.05 U instead of 0.15 U and a PCR volume of 25 µl instead of 35 µl. To detect amplification of nuclear paralogs of mtDNA, we used a mix of four half-nested PCR strategies applied on a dilution range of initial DNA template (pure DNA solution of 2 µl to 1/2500 dilution, primers in Suppl. material 1: Table S1 and Suppl. material 2: Table S2). We ran the first round PCRs with one step of 3 min at 95 °C; 37 cycles of 20 s at 95 °C, 30 s at 51 °C, 45 s at 72 °C; and one step of 5 min at 72 °C. The second round PCRs were run on 1 µl of the first round PCR product using the same parameters but 35 cycles. Service providers (BIOFIDAL, Vaulx-en-Velin, France) performed Sanger sequencing using PCR amplification primers.

We performed PCRs for 28S fragments at EZ LAB on the three specimens and at LEHNA on specimens AS1 and AS2. At EZ LAB we performed PCRs in 25 µl final volume using H2O: 18.3 µl, 10X Buffer: 2.5 µl, dNTPS (20 mM): 0.5 µl, Primer (10 µM): 0.6 µl forward and 0.6 µl reverse, polymerase (Eurobiotaq 5U/µl): 0.25 µl, and BSA (10 mg/ml): 0.25 µl (primers in Suppl. material 1: Table S1 and Suppl. material 2: Table S2). We applied the following PCR settings: one step of 3 min at 94 °C; 36 cycles of 45 s at 94 °C, 1 min at 47 °C, 1 min at 72 °C; and one step of 3 min at 72 °C. PCR products were sent to Macrogen Europe (Amsterdam, the Netherlands) for sequencing. At LEHNA, we amplified 28S fragments with 2 independent pairs of primers (Suppl. material 1: Table S1 and Suppl. material 2: Table S2) using the following settings: one step of 3 min at 95 °C; 37 cycles of 30 s at 95 °C, 30 s at 62 °C, 30 s at 72 °C; and one step of 5 min at 72 °C. BIOFIDAL performed Sanger sequencing. We visualized all chromatograms using FinchTV (Geospiza, Seattle, WA, USA). Sequences were submitted to GenBank (accession numbers OM640761–OM640764; OM501360–OM501366, Suppl. material 3: Table S3).

Molecular species delimitation

Species and higher taxa and their associated names represent hypotheses of independently evolving lineages that should ideally be supported by different lines of evidence (de Queiroz 2007; Padial et al. 2010; Fišer et al. 2018). In addition to morphological evidence, we used a COI-based molecular species delimitation approach to corroborate whether *A. ismailsezarii* sp. nov. was indeed a new species. In effect, we follow the diagnosable interpretation of the phylogenetic species concept (de Queiroz 2007).

COI sequences of *A. ismailsezarii* sp. nov. produced in this study were analysed with all COI sequences of taxa belonging to the "*Asellus* pattern" (*sensu* Henry and Magniez 1995) available in NCBI (Sequence extraction date: 25 August 2021). In addition to sequences of *A. ismailsezarii* sp. nov., we retrieved 581 sequences belonging to the *A. aquaticus sensu lato* species complex (including *A. kosswigi*, Verovnik et al. 2005, 2009; Sworobowicz et al. 2015, 2020; Lafuente et al. 2021), *Asellus* sp. from Ukraine, *Asellus hilgendorfii* from Japan, and two species from Lake Baikal, Russia – *Mesoasellus dybowskii* and *Limnoasellus poberezhnii* (*Nomen nudum* in Hidding et al. 2003) (Suppl. material 3: Table S3). We aligned the sequences with ClustalO in Seaview (Gouy et al. 2010) (Suppl. material 4: Table S4). We checked visually for the presence of anomalies, including stop codons and frameshifts, and removed ambiguous sites with the embedded Gblocks (with options for less stringed selection enforced) (Castresana 2000). COI maximum-likelihood phylogenies were reconstructed using PhyML (Guindon et al. 2010) under the GTR + G + I model of substitution with 4 substitution rate categories and a gamma shape parameter ($\alpha = 0.519$) as well as a proportion of invariant sites (0.392) estimated by maximum likelihood in PhyML. This model of evolution was previously used in several studies for delimiting species and reconstructing phylogenetic relationships among asellids (Lefébure et al. 2006; Morvan et al. 2013; Eme et al. 2018). We used MEGA (Kumar et al. 2018) as well, to find the best nucleotide substitution model and the optimal partitioning scheme for each marker. Molecular operational taxonomic units

(MOTUs) were delimited using the fixed threshold method implemented by Lefébure et al. (2006) for crustaceans. This method is based on the observation made from 1500 COI sequences belonging to 276 species of crustaceans that two clades diverging by more than 0.16 substitution per site, as measured by patristic distances, have a strong probability (ca. 0.99%) of belonging to different species. That method is conservative insofar as it identifies both fewer MOTUs and MOTUs that are more divergent than other delimitation methods, thereby limiting the risk of considering two populations of the same species as belonging to two distinct MOTUs. Several studies (Morvan et al. 2013; Sworobowicz et al. 2015; Eme et al. 2018) showed that evolutionary units of Asellids as delimited by distance-based methods, including that implemented by Lefébure et al. (2006), were further subdivided by tree-based methods such as the General Mixed Yule-Coalescent method (Pons et al. 2006) and the Poisson tree processes (Zhang et al. 2013). Our conservative estimate avoids potential oversplitting.

We used the R package "ape" (Paradis et al. 2004) to compute patristic distances and "cluster" to delimit MOTUs (Maechler et al. 2012).

To further assess the genetic differentiation of *A. ismailsezarii* sp. nov., we reconstructed the most likely phylogenetic relationships among taxa belonging to the "*Asellus* pattern" using all 28S sequences available in NCBI. Non-overlapping sequences (accession number KX467625) and short sequences (less than 300 bp, accession numbers AY739195 and HG322482) were excluded from the analyses. In addition to sequences of *A. ismailsezarii* sp. nov., we obtained 23 sequences belonging to the *A. aquaticus sensu lato* species complex (including *A. kosswigi*), *Asellus* sp. from Ukraine and *A. hilgendorfii* from Japan (Suppl. material 3: Table S3). As part of this study, we produced 28S sequences for *M. dybowskii* and *L. poberezhnii* using protocols described above for *A. ismailsezarii* sp. nov. We aligned sequences in Seaview with MAFFT (Katoh and Standley 2013) under the e-insi setting and removed ambiguous sites with the embedded Gblocks (with default options) (Suppl. material 5: Table S5). We reconstructed most likely relationships in the same way as for the partial COI gene.

Results

Taxonomy

Asellidae Rafinesque, 1815
***Asellus* E. L. Geoffroy, 1762**

***Asellus ismailsezarii* Malek-Hosseini, Jugovic, Fatemi & Douady, sp. nov.**
http://zoobank.org/5AD48986-0231-4775-9D8F-6F0D30169382
Figs 2–7

Material examined. *Holotype*: mature male, body length (BL) 8.2 mm (TU-SP.1), Ganow spring, Tuveh village, Andimeshk, Khuzestan Province, Iran, (32°48'31"N, 48°43'32"E, altitude 470 m above sea level). coll. Yaser Fatemi; 01. June.2019.

***Paratypes*:** 2 mature males, BL 8.2 mm (TU-SP.2), BL 6.5 mm (TU-SP.5), 1 juvenile male, BL 7.3 mm (TU-SP.7); 3 mature females, BL 8.1 mm (TU-SP.3), BL 8.1 mm (TU-SP.4), BL 6.7 mm (TU-SP.6); data same as holotype. ***Other material*:** 17 juvenile specimens (including specimens AS2 and AS3), data same as holotype. 1 mature female, BL 8.6 mm (specimen AS1), 01. December.2018, Same locality as holotype. coll. Mohammad Javad Malek-Hosseini and Yaser Fatemi. All material was preserved in 70% ethanol and deposited at UCBLZ (University Claude Bernard Lyon-Zoology, Lyon, http://cerese.univ-lyon1.fr/), under deposit numbers 2012.11.23.91–2012.11.23.99 and 2012.11.24.1–2012.11.24.47. We also deposited tubes containing stubs onto which pleopods I and II of holotype and paratype were mounted for producing SEM images.

Diagnosis of male. Species of *A. ismailsezarii* sp. nov. with depigmented body and complete loss of eyes (Fig. 2). Body elongated oval, more than three times (3.2–3.5) as long as wide. Head up to 1.4 times as wide as long; frontal margin bisinuate, medially concave, lateral margins rounded, each with posterolateral prominence with few weak setae. Pereonites I–III with slightly convex lateral margins, and pereonites IV–VII with straight lateral margins, with only few small simple short setae of about the same length. Pleomere I–II width about 60% of pereonite VII width. Pleotelson subrectangular, lateral margins densely fringed with simple short setae of about the same length, terminal edge rounded, with only weak median prominence. Antennae I and II, lengths about 15% and 70% of body length and with 8 and 55 flagellar articles, respectively. Propodus I slender ovoid, with weak apophysa on palmar margin. Carpus and propodus IV only slightly curved, superior margin and submarginal surface of propodus IV with few (6) short and weak simple setae. Length of longest spiniform

Figure 2. Habitus of *Asellus ismailsezarii* sp. nov. (Female AS1). The photo shows a specimen exposed to air after the stone it was under had been turned over. Body length is 8.6 mm.

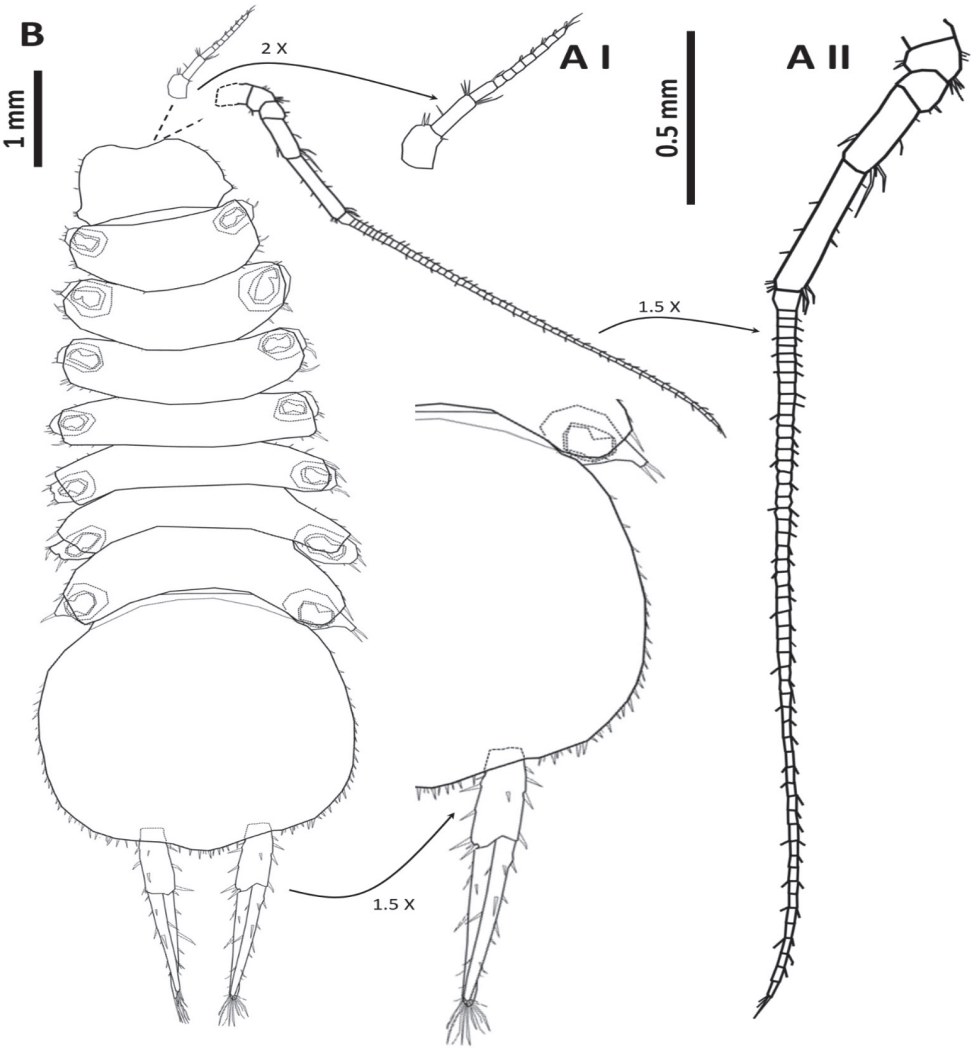

Figure 3. *Asellus ismailsezarii* sp. nov., Ganow spring, Western Iran. Holotype male, 8.2 mm: **A I, II** antennae I and II **B** body dorsal view.

robust seta on propodus VII inferior margin only about 3% pereopod VII length. Pleopod I exopodite without symmetric concavity on lateral margin, lateral margin being rather straight to slightly convex. Pleopod II architecture following typical "*Asellus* pattern" sensu Henry and Magniez (1995). Its endopodite with short (around 25% of protopodite length) horn-shaped basal spur (*processus calcariformis*). Pleopod IV and V with small respiratory areae, linea areae beginning and ending at the distal exopodite margin. Uropod short, up to about a quarter of body length.

Description of holotype and male type material (values in parenthesis). Body (Fig. 3B) 3.4 (♂♂: 3.3–3.5 ♀♀: 3.3–3.8) times as long as wide, elongated oval,

without pigmentation. Head (Fig. 3B) width 1.3 (♂♂: 1.1–1.4; ♀♀: 1.3–1.4) times of its length. Frontal margin bisinuate, medially concave, without rostral process, lateral margins rounded, each with small posterolateral protuberance, with few small, short stiff setae. No eye structures visible.

Pereonites (Fig. 3B) I–III with straight to slightly convex lateral margins and weakly rounded antero- and posterolateral angles, slightly protruding forwards. Last three pereonites with antero- and posterolateral angles slightly protruding backwards. Pereonite VI widest. Anterolateral, lateral and posterolateral margins of pereonites almost bare, with only sparse short simple setae. Coxopods well developed, margins of all epimerae dorsally visible, last three the most prominent.

Pleomere I–II short but wide (Fig. 3B), their width about 62% of pereonite VII width, forming a stalk largely covered by posterior margin of pereonite VII. Pleotelson (Fig. 3B) subrectangular, its width 1.02 (♂♂: 1.02–1.13; ♀♀: 0.96–1.01) times of its length, terminal edge rounded, with only weak median prominence between uropods. Lateral margins in their anterior quarters with few short setae, other three quarters with 30 (♂♂: 26–30; ♀♀: 26–36) (left side of pleotelson) and 32 (♂♂: 25–32; ♀♀: 29–35) (right side of pleotelson) dense but minute spiniform setae of about the same length. Terminal edge with a series of 12 (♂♂: 11–15; ♀♀: 11–19) short setae between the uropods. Dorsal surface almost bare, without setation.

Antenna I length (Fig. 3AI) 15% (♂♂: 15–16%; ♀♀: 16–17%) of body length, with 3 peduncular segments. First segment robust, with curved superior (longer) and inferior (shorter) margin, other two segments cylindrical. Second segment 1.3 (♂♂: 1.26–1.33; ♀♀: 0.98–1.30) times as long as first and 1.9 (♂♂: 1.28–1.88; ♀♀: 1.43–2.08) times as long as third. Longest setae on segment 1 approximately as along as width of second article, and length of longest setae on segment 2 about as long as article 3. Flagellum of 8 segments (♂♂: 6–8; ♀♀: 7–8), with 5 (♂♂: 3–5; ♀♀: 1–4) aestetascs on distal segments. Aesthetascs shorter as their parallel segments.

Antenna II length (Fig. 3AII) 62% (♂♂: 50–62%; ♀♀: 80–81%) of body length, with 6 peduncular and 55 (♂♂: 32–55; ♀♀: 47–55) flagellar segments. Sixth peduncular segment 1.6 (♂♂: 1.39–1.61; ♀♀: 1.39–1.43) times as long as fifth, both with only short setae, long setae present only on superior distal angles. Flagellum length 77% (♂♂: 66–77%; ♀♀: 76–77%) of antenna II length.

Mandibulae (Fig. 4MdbL) robust: *Pars molaris* (molar process) U-shaped, with toothed margin. *Pars incisiva* (incisor) formed by few blunt cusps arranged in semicircle. Left *lacinia mobilis* with few cups and spine row of about 15 biserrate setae, the distal ones being longest. Palp of three segments. First palpal segment widest, with few simple setae distally. Second palpal segment 1.6 times longer than first, without setation along external margin, with two simple setae distalo-mesially, and few simple setae along internal margin. Third palpal segment around half as long as second, with row of about 20 robust biserrate setae along external margin. Maxilla I (maxillule; Fig. 4MxI) lateral lobe with few smooth (the outer spines cone-shaped) and few weakly serrate robust spines. Distal part of outer margin with 1 long slender seta. Mesial lobe with 4 robust long plumose setae. Maxilla II (Fig. 4MxII) lateral and middle lobe with 19 slender and simple, and 13 curved pectinate robust setae, respectively, mesial lobe

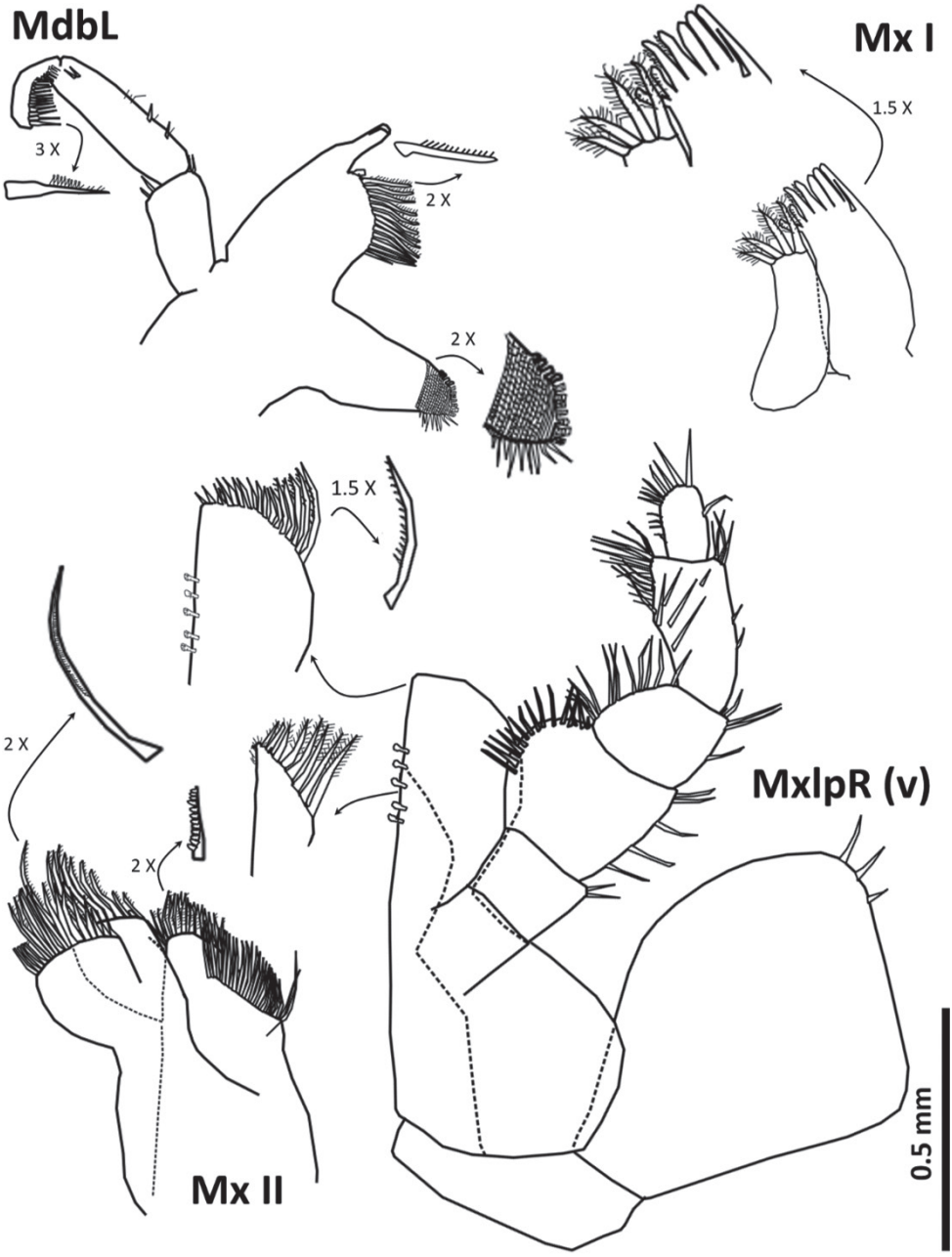

Figure 4. *Asellus ismailsezarii* sp. nov., Ganow spring, Western Iran. Paratype male, 8.1 mm (TU-SP. 2), mouthparts: MdL, left mandible; Mx I, maxilla I; Mx II, maxilla II, MxlpR (v), right maxilliped (ventrally).

with about 10 biserrate setae and parallel row of about 13 long simple setae along inner margin. Maxilliped (Fig. 4MxlpR) endite distal margin with about 11 biserrate robust setae, subapically with several rows of short simple setae. Mesial margin curved dorsally, with row of about 8 long biserrate setae, distomesial margin with setulose

fringe and around 5 coupling hooks. Palp of five articles. First article with 2 short setae apically on outer margin; second about 2.5 times as long as first, subtrapezoidal, with 5 long stiff setae on outer margin and row of about 15 longer medially directed simple setae on inner margin. Third article a bit shorter as second, less broad, with 4 long stiff setae on outer margin and row of about 11 slender simple setae on inner margin. Fourth article approximately twice as long as third, slender, distally wider, with a row of 6 and about 20 long slender setae along outer and inner margin, respectively. Fifth article as long as first, ovoid, fringed with around 10 long slender setae and 2 longest stiff simple apical setae. Epipodite subrectangular, lateral margin almost bare, with only few (3) short simple setae.

With the exception of the first and the fourth pair, seven pairs of pereopods similar in construction and ambulatory, increasing in length towards posterior pairs. Pereopod I (Fig. 5PpI) grasping, subchelate. Propodus I (article 6) slender ovoid, 2.2 (♂♂: 2.2–2.5; ♀♀: 2.6–2.8) times as long as wide, with weakly expressed proximal apophysa with few stronger spiniform setae (♂♂: 3–4; ♀♀: 2–4) and other sparsely set row of shorter simple setae (about 10 in total). Dactylus I (article 7) length about 70% (♂♂: 70–82%; ♀♀: 70–78%) of propodus length, with 5 (♂♂: 4–6; ♀♀: 5) sparsely placed slender stiff robust setae along inferior margin (their length increasing towards unguis). Pereopod I length 31% (♂♂: 31–38%; ♀♀: 31–40%) of body length, length relations of articles from ischium (article 2) to dactylus (article 7): 1: 0.9 (♂♂: 0.7–0.9; ♀♀: 0.7–1.0): 0.5 (♂♂: 0.3–0.5; ♀♀: 0.4–0.5): 0.2 (♂♂: 0.15–0.20; ♀♀: 0.20–0.24): 0.9 (♂♂: 0.7–0.9; ♀♀: 0.8–1.0): 0.7 (♂♂: 0.7–0.8; ♀♀: 0.7–0.8), unguis length 18% (♂♂: 18–25%; ♀♀: 19–24%) of dactylus length.

Pereopod IV (Fig. 5PpIV) grasping, with parallel, but only slightly curved superior and inferior margins of propodus. Pereopod IV length 36% (♂♂: 36–47%; ♀♀: 42–47%) of body length, length relations of articles from ischium (article 2) to dactylus (article 7): 1: 0.6 (♂♂: 0.6–0.8; ♀♀: 0.6–0.7): 0.4 (♂♂: 0.4–0.5; ♀♀: 0.4–0.5): 0.7 (♂♂: 0.7–0.9; ♀♀: 0.7–0.8): 0.8 (♂♂: 0.8–0.9; ♀♀: 0.8–1.0): 0.4 (♂♂: 0.3–0.4; ♀♀: 0.3–0.4), unguis length 32% (♂♂: 32–39%; ♀♀: 29–41%) of dactylus length. Carpus IV superiodistal angle with 5 (♂♂: 5–6; ♀♀: 5–6) spiniform setae, longest one 26% (♂♂: 18–26%; ♀♀: 17–26%) of carpus length. Propodus IV inferior margin and mesial surface with 6 (♂♂: 3–6; ♀♀: 3–4) acute stiff robust setae, longest robust seta 7% (♂♂: 7–10%; ♀♀: 8–11%) of propodus length, inferodistal surface with 3 (♂♂: 2–4; ♀♀: 0–5) short simple and penicilate setae, superior margin and submarginal surface with 6 (♂♂: 6–13; ♀♀: 12–13) short simple and penicilate setae, superior distal angle with 4 (♂♂: 4–6; ♀♀: 5–6) long simple setae and 1 penicilate seta. Dactylus IV inferior margin with 2 (♂♂: 2; ♀♀: 1–2) robust stiff setae, superior margin distally with 2–5 simple setae.

Pereopod VII (Fig. 5PpVII) with long slender articles, its length 70% (♂♂: 67–81%; ♀♀: 65–72%) of body length, length relations of articles along pereopod VII (given as in pereopod IV): 1: 0.9 (♂♂: 0.9–1.0; ♀♀: 0.8–0.9): 0.7 (♂♂: 0.6–0.7; ♀♀: 0.6–0.7): 1 (♂♂: 1.0–1.1; ♀♀: 0.9–1.1): 1.4 (♂♂: 1.2–1.4; ♀♀: 1.1–1.4): 0.3 (♂♂: 0.3–0.4; ♀♀: 0.3), unguis length 30% (♂♂: 30–39%; ♀♀: 33–43%) of dactylus length. Ischium VII with 8 (♂♂: 6–8; ♀♀: 6–6) spiniform setae along its margins,

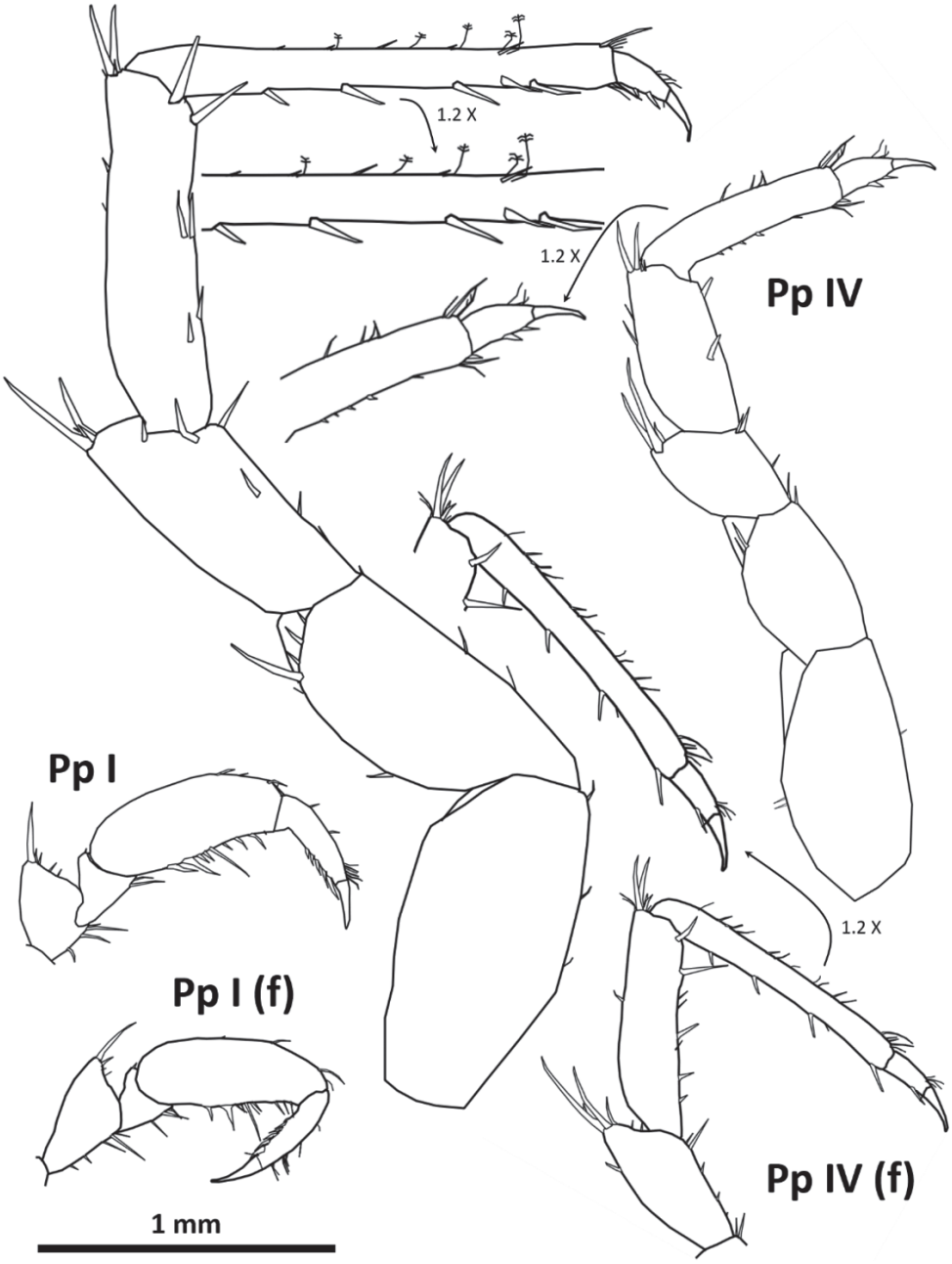

Figure 5. *Asellus ismailsezarii* sp. nov., Ganow spring, Western Iran. Holotype male, 8.2 mm: Pp I, IV, VII – pereopods I (distal articles), IV, VII. Female, 8.1 mm: Pp I, IV (f) – distal articles of pereopods I, IV.

with around 7 spiniform setae along inferodistal margins of merus, and a group of around 4 spiniform setae on superiodistal angle of merus VII, longest one 42% (♂♂: 42–58%; ♀♀: 44–53%) of merus length. Carpus VII with around 6 strong spiniform setae along inferior and inferodistal margins, few (around 2) weaker spiniform setae

along superior margin, and a group of (around two strong and two weak) spiniform setae at superior-distal angle. Propodus VII inferior margin with row of 6 (♂♂: 6; ♀♀: 6) acute stiff robust setae, longest robust seta 10% (♂♂: 9–11%; ♀♀: 11–15%) of propodus length, mesial surface and inferodistal angle with 6 (♂♂: 2–6; ♀♀: 3–5) submarginal simple setae, with few weak simple setae at inferodstal angle. Superior margin and submarginal surface with 10 (♂♂: 7–10; ♀♀: 3–12) short simple and penicilate setae, superior distal angle with 1 (♂♂: 1–2; ♀♀: 1–2) simple setae and around 5 penicilate seta. Dactylus VII inferior margin with 2 (♂♂: 2; ♀♀: 1–2) robust stiff setae, longest 30% (♂♂: 30–39%; ♀♀: 33–43%) of dactylus length, superior margin distally with around 5 (♂♂: 4–6; ♀♀: 4–5) simple setae.

Male pleopod I (Fig. 6PlpI, Fig. 7) protopodite 0.92 (0.88–0.96) times as wide as long, retinacle on medial margin of 5 (4–5, exceptionally less) hooks. Exopodite elongated ovoid, its width 77% (57–77%) of its length, with 19 (6–23) simple setae along lateral and distal margins, and 7 (7) plumose terminal marginal setae. Without concavity on lateral margin, lateral margin being rather straight to slightly convex. In juvenile males (Fig. 6PlpI (juv)), pleopod I exopodit external margin proximally elongated in a finger-like projection, with one plumose seta on it, and around 8 plumose setae along lateral and distal margins.

Male pleopod II (gonopod; Fig. 6PlpII, Fig. 7) protopodite subtrapezoidal, with rounded angles, its width 77% (76–83%) of its length. Lateral and medial margins without setae. Exopodite oval, almost twice (1.76–1.96) as long as wide, its proximal segment with around 2 simple setae on it its lateral margin; lateral and medial margins of distal segment fringed with 11 (6–11) simple and 6 (0–6) long plumose setae, respectively. A well-developed and rounded catch lobe on the dorsal side and medial margin of the distal segment of exopodite. This catch lobe partly overlaps the proximal segment of exopodite. Endopodite elongated ovoid, its length 60% (60–71%) of protopodite length, with a short horn-shaped basal spur (*processus calcariformis*): 43% (37–55%) of endopodite length. Dorsal side of endopodite entirely smooth. Ventral side with a finger-like labial spur (*processus cylindriformis*), an anterior lobe densely covered with serrated scales, and a spoon-shaped *Capitulum.* Surface of *Capitulum* is smooth and without spines. A short but large-diameter *Cannula* is clearly visible with scanning electron microscopy between the anterior lobe and *Capitulum*. In juvenile males, pleopod II (Fig. 6PlpII (juv)) without endopodite, exopodite almost without setation.

Pleopod III (Fig. 6PlpIII) exopodite rounded triangular, about 1.6 times as long as wide, with almost straight medial margin. Medio-distal, terminal and latero-distal margins with around 8 long plumose setae. No setation along medial and lateral margins. Endopodite length about 0.7 of exopodite length.

Pleopod IV (Fig. 6PlpIV) exopodite broadly ovoid, about 1.6 times as long as wide, its area equally shaped as in pleopod V. Without setation along margins. Endopodite subrectangular, its length about 0.8 of exopodite length.

Pleopod V (Fig. 6PlpV) exopodite ovoid, 1.6 (♂♂: 1.6–1.7; ♀♀: 1.5–1.6) times as long as wide, its margins without setation. Respiratory area small, its surface 21% (♂♂: 21–28%; ♀♀: 22–28%) of exopodite surface, linea areae beginning and ending

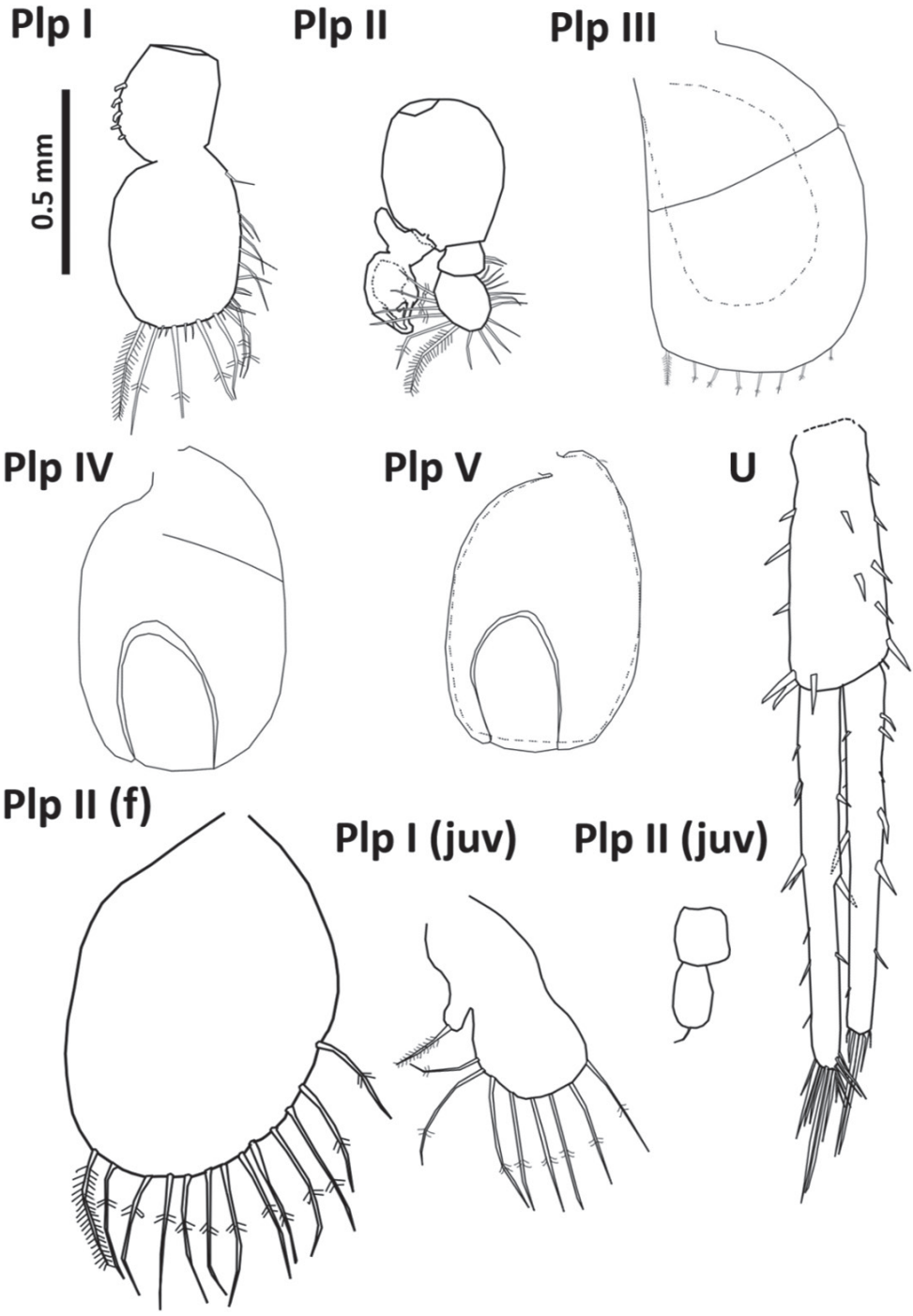

Figure 6. *Asellus ismailsezarii* sp. nov., Ganow spring, Western Iran. Holotype male, 8.2 mm: Plp I, II; II, IV, V – pleopods I, II, III, IV, IV; U – uropod (pleopod VI). Female, 8.1 mm: Plp II (f) – pleopod II. Juvenile male, 7.3 mm: Plp I, II (juv) – pleopods I, II.

on the distal exopodite margin. Endopodite suboval, its length almost as long (holotype: 97%) as exopodite.

Uropod (Fig. 6U) length 24% (♂♂: 24–25%; ♀♀: 17–24%) of body length. Proto-, endo- and exopodite length relations: 1:1.52 (♂♂: 1.40–1.52; ♀♀: 1.53–1.77):1.49 (♂♂: 1.25–1.49; ♀♀: 1.30–1.56). Endopodite with 9 (♂♂: 9; ♀♀: 8–9) spiniform simple setae and 7 (♂♂: 7–9; ♀♀: 8–9) penicilate setae, longest simple seta length 14% (♂♂: 14–15%; ♀♀: 10–13%) of endopodite length.

Female type material. Body length 6.7–8.1 mm (3.3–3.8 times of body width), almost identical to male except, antenna II seems longer than in males, around 4/5 of body length, but with similar number of flagellar segments (47–55). Pereopod I (Fig. 5PpI(F)) propodus with less expressed proximal apophysa, slender and longer propodus, 2.6–2.8 times as long as wide (♂♂: 2.2–2.5). Pereopod IV (Fig. 5PpIV(f)) not for grasping, ambulatory, little longer than the preceding pairs. Without pleopod I. Pleopod II (Fig. 6PlpII(f)) suboval, about 1.3 (1.3–1.4) times as long as wide, with 12–13 long marginal plumose setae. Uropods seem a bit shorter than in males (♂♂: 24–25%; ♀♀: 17–24%), with relatively longer endopodite compared to protopodite (♂♂: 1.40–1.52 *versus* ♀♀: 1.53–1.77 times as long as protopodite).

Morphological comparison with other *Asellus* species. Among the 18 species of *Asellus* presently described, *A. ismailsezarii* sp. nov. resembles morphologically *A. monticola* Birstein, 1932 (Suppl. material 6: Table S6). Pleopods I and II appear to be almost identical between the two species, although Henry and Magniez (1996) did not provide SEM photos of these pleopods for *A. monticola*. In both species, the endopodite of pleopod II has a spoon-shaped *Capitulum* with no spines. However, *A. ismailsezarii* sp. nov. differs from *A. monticola* in that it is eyeless and fully depigmented, has a slightly curved pereopod IV and bears no setae on proximal margins of exopodite of pleopods IV and V. These morphological differences are sufficient to consider the studied specimens as belonging to a distinct species. We also provided in Suppl. material 6: Table S6, a morphological comparison with *A. kosswigi*, *A. aquaticus aquaticus*, *A. aquaticus infernus* and *A. aquaticus cavernicolus* showing that *A. ismailsezarii* sp. nov. shows many morphological differences with both surface and subterranean aquatic species and subspecies of the *A. aquaticus* complex. Contrary to *A. ismailsezarii* sp. nov., all members of the *A. aquaticus* complex show a helical *Capitulum*, suggesting the new species does not belong to that complex.

Molecular species delimitation. We found eleven MOTUs within the "*Asellus* pattern", including one MOTU corresponding to *A. ismailsezarii* sp. nov. (Fig. 8A). Maximal patristic distances among COI sequences within the same MOTU (i.e., within-MOTU distances) ranged from 0 to 0.19 (0.01 for *A. ismailsezarii* sp. nov.) (Suppl. material 7: Table S7). Minimal patristic distances among sequences belonging to *A. ismailsezarii* sp. nov. and sequences belonging to the other MOTUs (i.e., between-MOTU distances) ranged from 0.76 to 1.00 (Suppl. material 7: Table S7). The considerable gap between within- and between-MOTU patristic distances supports our taxonomic assessment that *A. ismailsezarii* sp. nov. is a distinct species.

Phylogenetic relationships as inferred from the COI gene indicated that *A. ismailsezarii* sp. nov. does not belong to the *A. aquaticus* complex, which itself contained

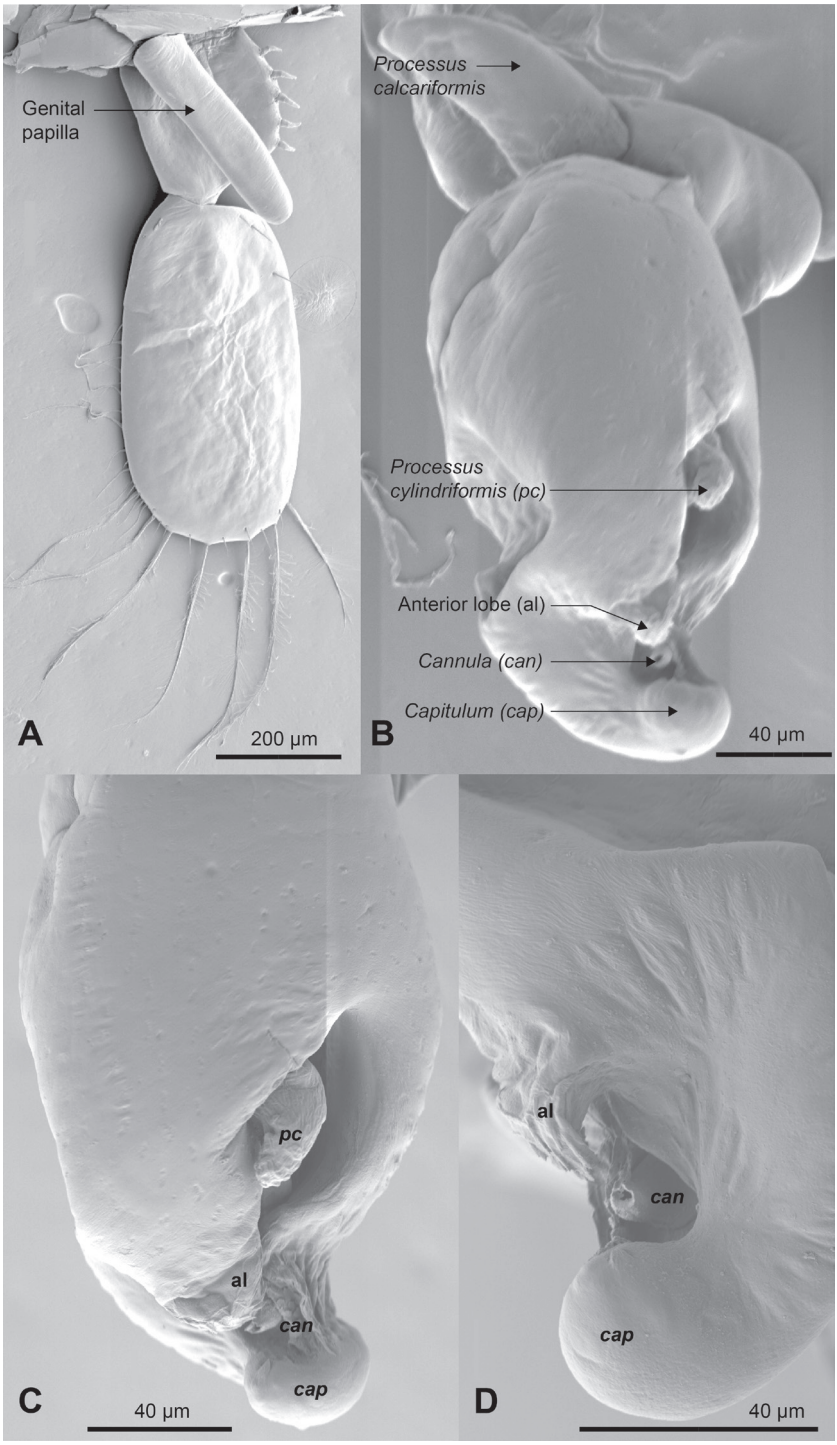

Figure 7. *Asellus ismailsezarii* sp. nov., male; SEM micrographs of (**A**) pleopod I (paratype male BL 8.2 mm: TU-SP.2). Photo also shows one genital papilla inserted on ventral side of pereionite VIII **B, C, D** pleopod II endopod (holotype male BL 8.2 mm: TU-SP.1) **B, C** ventral view and **D** lateral view.

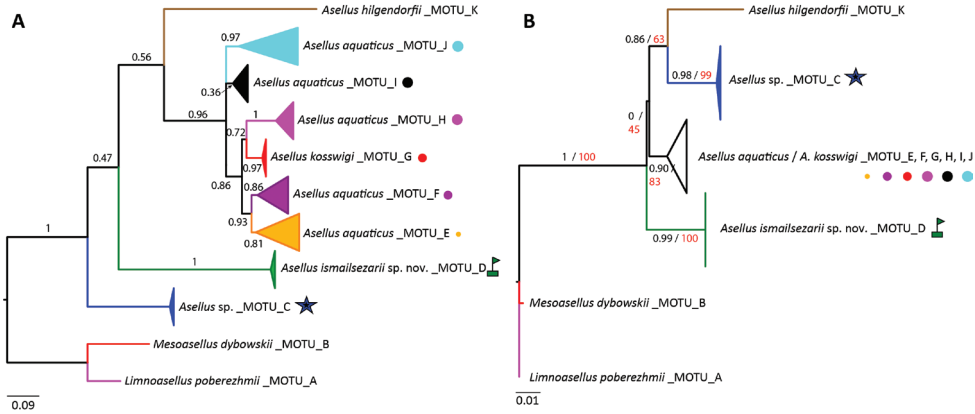

Figure 8. Phylogenetic relationships among taxa belonging to the *Asellus* pattern derived from Maximum likelihood analysis of (**A**) 585 COI mtDNA sequences, (**B**) thirty 28S rDNA sequences. The clade comprised of *M. dybowskii* and *L. poberezhnii* was used as outgroup. In A, branches to molecular operational taxonomic units (MOTU) as delimited with the fixed threshold method implemented by Lefébure et al. (2006) were collapsed. In B, branches to the main clades were collapsed. Colors and symbols as in Fig. 9. Branch supports are approximate likelihood ratio test (aLRT) values (black) and bootstrap values (red).

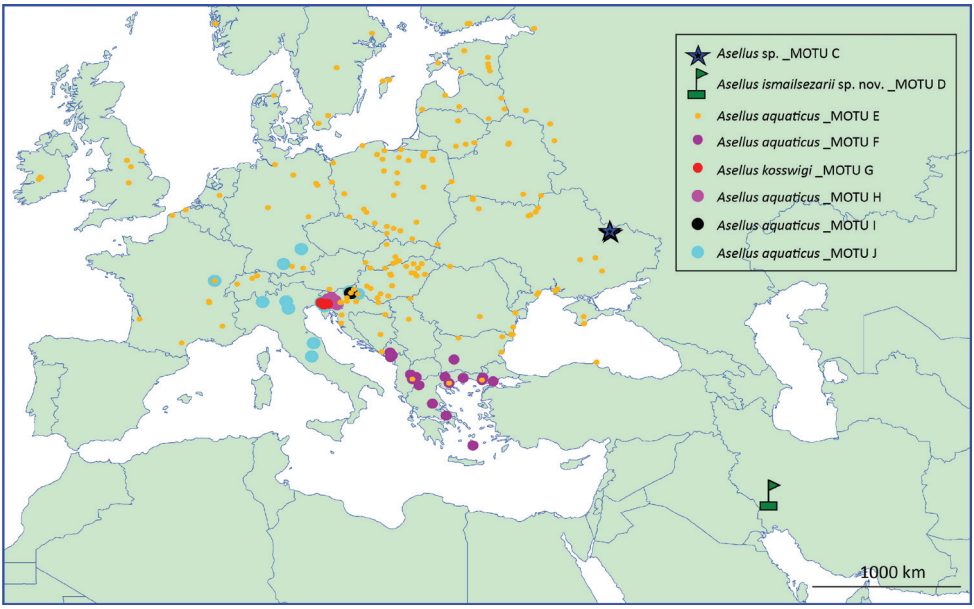

Figure 9. Map showing the distribution of molecular operational taxonomic units (MOTU) within the "*Asellus* pattern". Site locations for *Asellus hilgendorfii* (Japan) and *Mesoasellus dybowskii* and *Limnoasellus poberezhnii* (Lake Baikal, Russia) are not shown. Colors and symbols as in Fig. 8.

six MOTUs, including *A. kosswigi* (Fig. 8A). Within that complex, only MOTU E corresponding to *A. aquaticus* Linnaeus, 1758 *sensu stricto* was widely distributed across Europe (Fig. 9). The resulting tree of the nuclear 28S rDNA sequences agreed well with the COI phylogeny, again indicating that *A. ismailsezarii* sp. nov. was a sister species

of the *A. aquaticus* complex (Fig. 8B). Patristic distances derived from the 28S loci also corroborated COI results by revealing a strong divergence between *A. ismailsezarii* sp. nov. and the other MOTUs.

Etymology. The name of the new species is a patronym for ''Mohammad Ismail Sezari", the legendary guard of the train tracks from the area of the type locality. In year 1986, he sacrificed his life to save 750 others that were on a collision course train.

Discussion

The present study provides morphological and molecular evidence supporting the species status of *Asellus ismailsezarii* sp. nov. This newly discovered species shows diagnostic morphological features of the *Asellus* genus and *Asellus* subgenus (Magniez and Henry 1970; Henry and Magniez 1993, 1996). In addition to being eyeless and fully depigmented, *A. ismailsezarii* sp. nov. differs from the surface aquatic species *A. monticola* to which it is supposedly most closely related, by the shape of its pereopod IV and the lack of setation on pleopods IV and V. Most but not all groundwater species lack a grasping pereopod IV, suggesting adult males no longer engage in precopulatory mate guarding (Henry 1976). The occurrence of a still curved pereopod IV among some groundwater asellids could indicate that groundwater colonization has occurred recently. However, this hypothesis deserves to be tested using a large number of population and/or species (see for example Balázs et al. 2021). We also present morphological evidence that *Asellus ismailsezarii* sp. nov. is distantly related to species belonging to the *A. aquaticus* complex. All species of that complex are characterized by a helical *Capitulum*, whereas both *Asellus ismailsezarii* sp. nov. and *A. monticola* have a spoon-shaped *Capitulum.*

In addition to morphological evidence, we show that patristic distances for the COI gene between *A. ismailsezarii* sp. nov. and any other MOTUs delimited within the "*Asellus* pattern" largely exceed the 0.16 threshold value above which two clades can be considered to belong to distinct species (Lefébure et al. 2006). Phylogenetic relationships as inferred from the COI and 28S gene confirm that *A. ismailsezarii* sp. nov. does not belong to the *A. aquaticus* complex.

Our findings together with previous asellid reports from Iran and neighboring countries (Turkmenistan, Georgia, Armenia) suggest that the Caspian Sea region is a contact zone between species of the European *A. aquaticus* complex, including *A. aquaticus* Linnaeus, 1758 and *A. aquaticus messerianus* and Asian species, including *A. monticola* and *A. ismailsezarii* sp. nov. Although the number of MOTUs within the *A. aquaticus* complex varies according to the species delimitation method – Sworobowicz et al. (2015) recognized 13 MOTUs using the automatic barcode gap discovery – one MOTU corresponding to the neotype population of *A. aquaticus* Linnaeus, 1758 (see Verovnik et al. 2009) systematically shows a large distribution throughout Europe. According to records by Rémy (1941) and Henry and Magniez (1995), *A. aquaticus* Linnaeus, 1758 is also present in Iran. Further sampling and sequencing of asellids in the Caspian See region is necessary to ascertain the existence of that contact zone between species of the European *A. aquaticus* complex and Asian species.

The molecular systematics of the *Asellus* and more largely of the "*Asellus* pattern" is yet to be established since most of its species have not yet been sequenced. Hence, except from the fact that *A. ismailsezarii* sp. nov. does not belong to the *A. aquaticus* complex, its phylogenetic position within the *Asellus* remains to be more precisely determined. We expect that *A. ismailsezarii* sp. nov. is phylogenetically closely related to *A. monticola* because the two species share many morphological characters. This hypothesis can be tested when fresh material of *A. monticola* becomes available for DNA sequencing. Ancestral populations of *A. monticola* might have colonized groundwater on multiple occasions and localities. If so, many more obligate groundwater *Asellus* taxa could be discovered in Iran.

We have several reasons to expect many more groundwater species discoveries in Iran as sampling effort increases. First, the number of 43 obligate groundwater species presently known from Iran is exceedingly low considering the extent and diversity of groundwater habitats (Vardanjani et al. 2017). Of the 2000 registered caves and many more wells and springs in Iran, a tiny proportion has actually been sampled. Second, about 70% of the species were described in the past eight years when sampling and taxonomic efforts intensified. Third, the number of species is very unevenly distributed among taxa, reflecting a strong taxonomic bias in description effort. Of the 43 obligate groundwater species, 23 belong to the genus *Niphargus* Schiødte, 1847, due to a recent increase in species description activity among Iranian experts (Malek-Hosseini and Zamani 2017; Zamanpoore et al. 2020; Bargrizaneh et al. 2021). Yet, recent species descriptions of Stenasellidae, aquatic Isopoda (Khalaji-Pirbalouty et al. 2018), Moitessieriidae, aquatic snails (Fatemi et al. 2019), Agnaridae, terrestrial Isopoda (Kashani et al. 2013) and Carabidae, terrestrial Coleoptera (Malek-Hosseini et al. 2021) indicate that many animal groups are likely to have presently unknown subterranean representatives in Iran. Local but repeated sampling is also revealing the presence of potentially species-rich aquifers with high conservation value. The karst aquifer feeding the Ganow spring, the type locality of *A. ismailsezarii* sp. nov., also harbors three described species of cave fish (Mousavi-Sabet et al. 2016; Vatandoust et al. 2019) as well as yet-undescribed species of gastropods (in first author collection). This paper contributes a piece in the puzzle of the Iranian cave biodiversity.

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Supplementary material 1

Table S1. DNA extraction and PCR protocols

Authors: Mohammad Javad Malek-Hosseini, Jure Jugovic, Yaser Fatemi, Matjaž Kuntner, Rok Kostanjšek, Christophe J. Douady, Florian Malard

Data type: pdf. file

Explanation note: Molecular protocols indicating for each gene (COI and 28S) and specimen of *Asellus ismailsezarii* sp. nov. (AS1, AS2 and AS3) the DNA extraction protocol (see text), PCR protocol (see text), primer names, sequencing institutions and sequence codes.

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Link: https://doi.org/10.3897/subtbiol.42.79447.suppl1

Supplementary material 2

Table S2. List of primers

Authors: Mohammad Javad Malek-Hosseini, Jure Jugovic, Yaser Fatemi, Matjaž Kuntner, Rok Kostanjšek, Christophe J. Douady, Florian Malard

Data type: pdf. file

Explanation note: List of primers used in this study.

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Link: https://doi.org/10.3897/subtbiol.42.79447.suppl2

Supplementary material 3

Table S3. COI and 28S sequence data set with sampling localities

Authors: Mohammad Javad Malek-Hosseini, Jure Jugovic, Yaser Fatemi, Matjaž Kuntner, Rok Kostanjšek, Christophe J. Douady, Florian Malard

Data type: GenBank codes (excel file)

Explanation note: COI and 28S sequence data set used in the present study. TH: Molecular operational taxonomic units (MOTU) as delimited with the 16% COI divergence threshold; Long.: longitude of the locality, in decimal degrees; Lat.: latitude of the locality, in decimal degrees, There may be more than one locality per sequence in case hapoltype sequences were desposited to GenBank.

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Link: https://doi.org/10.3897/subtbiol.42.79447.suppl3

Supplementary material 4

Table S4. COI alignment

Authors: Mohammad Javad Malek-Hosseini, Jure Jugovic, Yaser Fatemi, Matjaž Kuntner, Rok Kostanjšek, Christophe J. Douady, Florian Malard

Data type: mase. file

Explanation note: COI alignment data.

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Link: https://doi.org/10.3897/subtbiol.42.79447.suppl4

Supplementary material 5

Table S5. 28S alignment

Authors: Mohammad Javad Malek-Hosseini, Jure Jugovic, Yaser Fatemi, Matjaž Kuntner, Rok Kostanjšek, Christophe J. Douady, Florian Malard

Data type: mase. file

Explanation note: 28S alignment data.

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Link: https://doi.org/10.3897/subtbiol.42.79447.suppl5

Supplementary material 6

Table S6. Morphological comparison with other *Asellus* species

Authors: Mohammad Javad Malek-Hosseini, Jure Jugovic, Yaser Fatemi, Matjaž Kuntner, Rok Kostanjšek, Christophe J. Douady, Florian Malard

Data type: pdf. file

Explanation note: Comparison of *Asellus ismailsezarii* sp. nov. with *A. monticola*, *A. kosswigi*, and *Asellus aquaticus* and its known hypogean subspecies from Europe (*A. a. infernus*, *A. a. cavernicolous*). Abbreviations: A II – antenna II, Prp IV, VII – pereopods IV and VII, Plp IV, V – pleopods IV and V. Most discriminative features between *A. ismailsezarii* and any other species analysed here are presented in bold (note value overlapping).

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Link: https://doi.org/10.3897/subtbiol.42.79447.suppl6

Supplementary material 7

Table S7. Genetic distances among and within MOTUs

Authors: Mohammad Javad Malek-Hosseini, Jure Jugovic, Yaser Fatemi, Matjaž Kuntner, Rok Kostanjšek, Christophe J. Douady, Florian Malard

Data type: excel file

Explanation note: Genetic distances among MOTUs. Letters correspond to MOTUs as shown in Figs 8 and 9. Below diagonal: Minimum patristic distances among MOTUs. Above diagonal: minimum uncorrected distances (p-distance) among MOTUs. Diagonal: maximum patristic distances within MOTUs / maximum uncorrected distances (p-distance) within MOTUs.

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RESEARCH ARTICLE

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Selachochthonius naledi sp. nov. (Pseudoscorpiones, Pseudotyrannochthoniidae), a new troglobitic species from South Africa

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Abstract

A new pseudoscorpion species, *Selachochthonius naledi* **sp. nov.**, is herein described. It can be distinguished from the other species of the genus mainly by the absence of eyes, number and morphology of chelal teeth and coxae setae and by the appendices proportions. This new species represents the fourth record for the genus in South Africa. We also provide some ecological remarks of the new species and recommendations for future research.

Keywords

Cave-dwelling, pseudoscorpion, taxonomy

Introduction

Pseudoscorpions (Arachnida: Pseudoscorpiones) are small sized predatory arthropods distributed throughout terrestrial habitats commonly found across six continents (except for Antarctica). They occur in a diversity of habitats including leaf litter, beneath

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bark or stones, as well as bird nests or animal burrows. Several species are also recorded from caves and may exhibit subterranean-adapted traits (Chamberlin 1931; Harvey 1988; Harms 2018). These arachnids are highly diverse, comprising of 26 families and 465 genera (Benavides et. al. 2019; World Pseudoscorpiones Catalog 2021).

The family Pseudotyrannochthoniidae comprises 69 species distributed in five genera: *Allochthonius* Chamberlin, 1929 with 28 species, *Afrochthonius* Beier, 1930 with seven species, *Centrochthonius* Beier, 1931 with three species, *Pseudotyrannochthonius* Beier, 1930 with 28 species and *Selachochthonius* Chamberlin, 1929 with three species (Harms 2013; Harms and Harvey 2013; Viana and Ferreira 2021; World Pseudoscorpiones Catalog 2021).

This family is diagnosed by the presence of *ib* and *isb* trichobothria located at the base of the fixed finger and coxal spines present only in coxae I (Harms and Harvey 2013). While the Northern Hemisphere members of the family present a more variable carapacal chaetotaxy, the Southern Hemisphere species share the presence of 18 carapace setae (Schwarze et al. 2021). In South Africa, pseudotyrannochthoniid pseudoscorpions are represented by two genera, namely *Afrochthonius* and *Selachochthonius*, from which the latter can be identified mainly by the presence of an intercoxal tubercle (Ellingsen 1912; Beier 1930).

During an expedition to caves in South Africa (carried out 5–17 October 2019), two specimens of Pseudotyrannochthoniidae pseudoscorpions were found, belonging to a new species herein described. We also provide some notes on its habitat and potential threats, conservation issues, a brief discussion on its association with the cave environment and a comparison of morphological features with all *Selachochthonius* species, presenting their distribution in South Africa.

Materials and methods

Study area

The study areas included two cave systems, namely Villa Louisa (26°01'25.5"S, 27°42'43.0"E) and Yom Tov (26°01'14.92"S, 27°42'53.46"E) caves (Fig. 1A–C), located in the Cradle of Humankind UNESCO World Heritage Site (Gauteng Province, South Africa). The landscape is associated with the Rocky Highveld Grassland, which generally supports a high diversity of species. Rain often occurs as thunderstorms during the hot summer months with an average of 700 mm per year. The caves are mainly developed in dolomitic bedrock, which form part of the Monte Christo Formation (Malmani Subgroup, Transvaal Supergroup) (Dirks et al. 2015).

Field sampling

Fieldwork was conducted in October 2019. This trip was part of a worldwide scale project (with samplings in all continents, except Antarctica) aiming to evaluate how habitat traits influences invertebrate communities (manuscript in prep.). In South Africa, seven caves were sampled. Untimed direct intuitive searches (*sensu* Wynne et al.

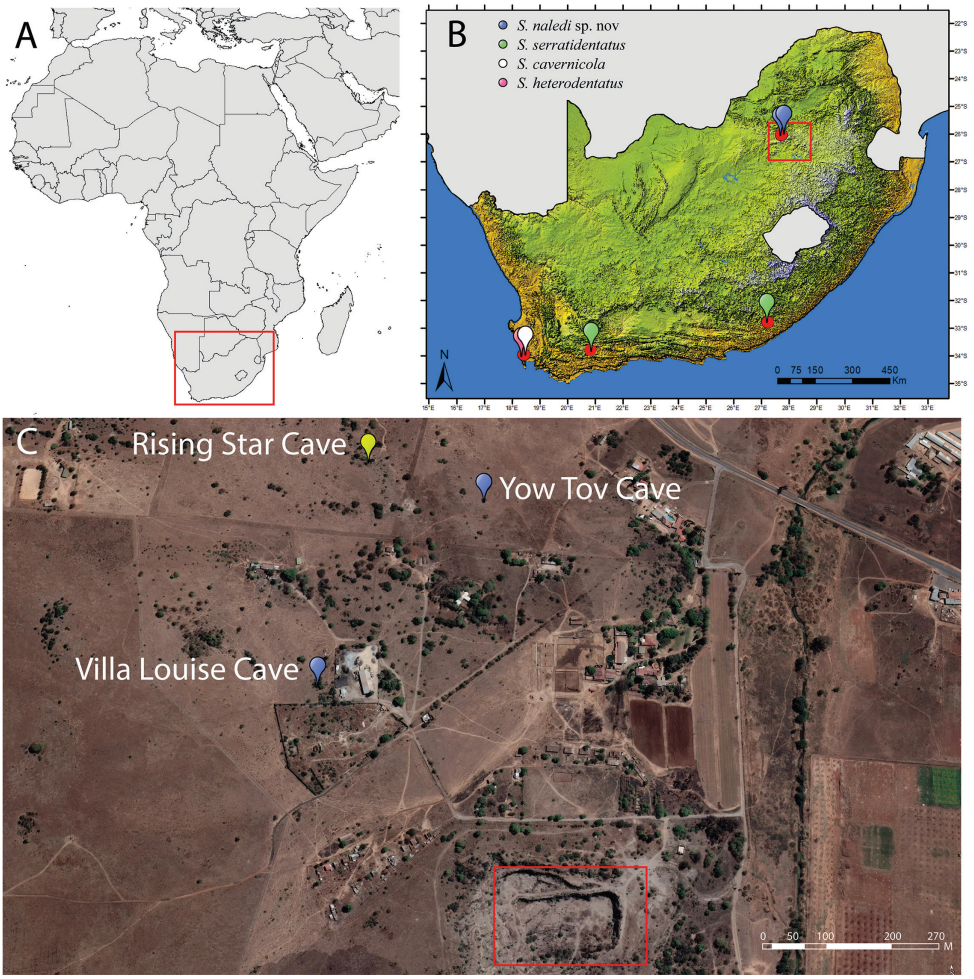

Figure 1. Distribution map of *Selachochthonius* species in South Africa **A** Africa continent, detail on South Africa country **B** South Africa, exhibiting distribution of *Selachochthonius* species with detail on *S. naledi* sp. nov. type localities **C** *Selachochthonius naledi* sp. nov. distribution area, exhibiting Yom Tov, Villa Louise and Rising Star Caves (*Homo naledi* type locality), detail on quarry activity occurring nearby.

2019) were used within multiple 10×3 m transects. The number of sampled transects was proportional to the cave size. Opportunistic sampling was also carried out to collect arthropods prioritizing organic deposits and microhabitats. All invertebrates were collected with a fine brush and stored in 70% ethanol.

Analysis and preparation

To properly examine taxonomic characters, specimens were photographed, dissected and mounted on temporary cavity slides with glycerine. Photographs and measurements were taken using a Zeiss Axio Zoom V16 stereomicroscope and ZEN 2.3

software package. Appendices and structures were mounted on Kaiser's glycerol gelatine for drawings, due to its stabilisation and low temperature solidification. Drawings were prepared with a drawing tube on an Olympus BX40 optical microscope equipped with phase contrast. Following, illustrations were vectorized using Inkscape 1.1 software package (Montesanto 2015; inkscape.org). The holotype and paratype were deposited in the National Collection of Arachnida (NCA), Pretoria, South Africa.

Terminology

Terminology follows Chamberlin (1931), Harvey (1992) and Judson (2007). Abbreviations for the trichobothria: *b* = basal; *sb* = sub-basal; *st* = sub-terminal; *t* = terminal; *ib* = interior basal; *isb* = interior sub-basal; *ist* = interior sub-terminal; *it* = interior terminal; *eb* = exterior basal; *esb* = exterior sub-basal; *est* = exterior sub-terminal; *et* = exterior terminal. Abbreviation for repository: NCA – National Collection of Arachnida.

Results

Family Pseudotyrannochthoniidae Beier, 1932
Genus *Selachochthonius* Chamberlin, 1929

***Selachochthonius naledi* sp. nov.**
http://zoobank.org/8BD115F5-1B22-4EC7-919F-92007DCD91D1

Material examined. ***Holotype*** female (NCA 2021/1351), preserved in ethanol: South Africa, Cradle of Humankind (UNESCO WHS), Maquassi Hills Municipality, Villa Louisa cave (26°01'25.5"S, 27°42'43.0"E), 09 October 2019, leg. R.L Ferreira. ***Paratype*** male (NCA 2021/1352), Cradle of Humankind, Maquassi Hills Municipality, Yom Tov Cave (26°01'14.92"S, 27°42'53.46"E), 17 October 2019, leg. R.L Ferreira.

Etymology. The epithet *naledi* refers to *Homo naledi*, an extinct hominid species discovered within the Dinaledi chamber. This chamber is located in the Rising Star Cave (Fig. 1C), a World Heritage site located approximately 50 km from Johannesburg. The caves where the pseudoscorpions were found are located on the same hill as Rising Star Cave. The distances between the entrance of Rising Star Cave and the entrances of Vila Louise and Yom Tov caves are approximately 425 and 220 meters, respectively. Considering the fissure system associated with the carbonatic rock in the area, it is likely that the pseudoscorpions are able to disperse through small fissures to other macrocaves in the hill, including Rising Star Cave.

Diagnosis. *Selachochthonius naledi* sp. nov. differs from other members of the genus by the following combination of characters: absence of eyes or eyespots (*S. heterodentatus* Beier, 1995 with four well-developed eyes and *S. serratidentatus* Ellingsen, 1912 bearing four small eyes) (Ellingsen 1912; Beier 1955); epistome triangular and strongly dentate (*S. cavernicola* Lawrence, 1935 presents a flattened apex and slightly dentated

epistome) (Lawrence 1935); palpal femur with length of 0.65–0.66 mm or 6.2 times longer than wide (4.0 times in *S. serratidentatus* and *S. heterodentatus* with femur length of 1.0 mm); chela 6.1–6.2 times longer than wide (3.8 times in *S. serratidentatus* and 5.0 times in *S. heterodentatus*) (Ellingsen 1912; Beier 1955); fixed finger with 32–34 simple, sparse, acute and triangular teeth (*S. cavernicola* with 23 sparse, simple and triangular teeth, presented in only 2/3 of the segment; *S. heterodentatus* with 28 acute teeth intercalated by 24 smaller teeth and *S. serratidentatus* with sparse, acute and complex teeth, each one intercalated by one or two smaller teeth) (Ellingsen 1912; Lawrence 1935; Beier 1955); movable finger with 29–31 simple, sparse acute and triangular teeth (*S. cavernicola* with 17 simple, sparse, acute and triangular teeth; *S. heterodentatus* with 16 acute and triangular, intercalated by 12 smaller teeth) (Lawrence 1935; Beier 1955); presence of a small tubercle in both chelae between the 13th and 14th teeth of the female movable finger and between the 10th and 11th teeth of the male movable finger (*S. cavernicola*, *S. heterodentatus* and *S. serratidentatus* lacks tubercle); 7–11 coxal spines tripinnate arranged in a single row on the anterior portion of the coxa I (*S. cavernicola* with 12 bipinnate coxal spines) (Lawrence 1935).

Description. (Fig. 5E–F). Body pale yellowish, mostly translucent; chelicerae slightly reddish orange, abdomen beige. Some parts of the body scaly. Vestitural setae sharp and anteriorly projected.

Carapace. (Fig. 2B–D). Ratio length/width near 1.0, strongly constricted posteriorly showing a difference between ocular width and posterior width of 0.08–0.10 mm; anterior margin smooth; absence of eyes or eyespots; epistome strongly dentate and saw-like; posterior margin of carapace smooth; chaetotaxy 6: 4: 4: 2: 2 (18), lateral pre ocular setae are shorter in female (Fig. 2C).

Chelicera. (Fig. 2A). Hand with 7–8 setae; movable finger with 1 subdistal seta and fixed finger with 1 subdistal setae as well; galea absent; fixed finger with 11–12 acute teeth; movable finger with 8–9 acute teeth including one large distal tooth; rallum with 11 blades, middle blades long; serrula exterior with 22–25 blades, serrula interior with 19–21 blades.

Tergites. Not divided; surface smooth; chaetotaxy uniseriate, I–XI 2: 2: 4: 4: 4–6: 6–8: 6: 4–6: 4: 4: 4. Anal operculum with two dorsal setae. Pleural membrane smooth.

Coxae. (Fig. 3A–C). Manducatory process with two apical setae (the distal one about half of the length of the proximal one); rest of palp coxae with 3 setae arranged in a triangle; delicate lamellae outlined by 15 small spines. Pedal coxae (Fig. 3A–C): coxal spines tripinnate, with smooth tips arranged in a single oblique row in coxae I (7–11), chaetotaxy: I 4, II 4, III 4–5, IV 5 intercoxal tubercle present reduced between coxae III and IV, female bearing two setae and the male one.

Genital operculum of female: setae distributed in three horizontal rows: 2: 2: 3, genital opening not bifurcated (horizontally contiguous).

Genital operculum of male (Fig. 3D): setae distributed in two horizontal rows: 2: 2; Anterior genital operculum with 10 setae; genital opening with 8–9 valvular genital setae.

Sternites. chaetotaxy IV−XI: 10–12: 12–14: 6–12: 6–8: 6: 6: 6: 4. Anal operculum with two ventral setae.

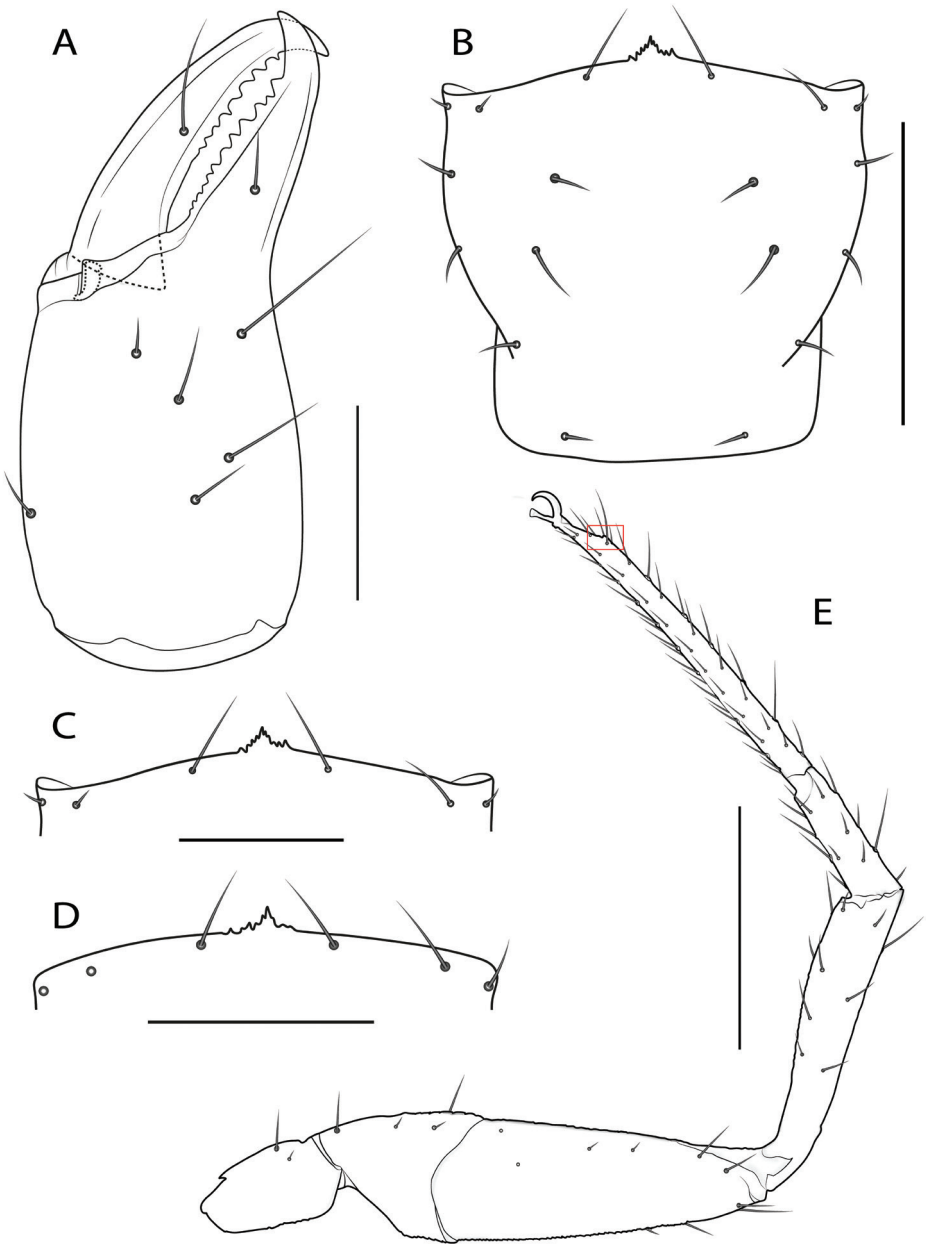

Figure 2. *Selachochthonius naledi* sp. nov. **A** left chelicera of female holotype, antiaxial view **B** female holotype carapace, showing chaetotaxy of carapace **C** detailed anterior margin of female paratype carapace **D** detailed anterior margin of male paratype carapace **E** leg IV of female holotype, showing tubercle location. Scale bars: 0.25 mm (**A, C–D**); 0.5 mm (**B, E**).

Palp (Fig. 4A–D). Trochanter 1.4–1.8 times longer than wide, patella 2.3–2.4 times longer than wide, femur 6.2–6.6 times longer than wide. Femoral chaetotaxy 3: 4: 4–5: 2: 7: 1. Trichobothrial pattern: *ib* and *isb* located at the distal portion of

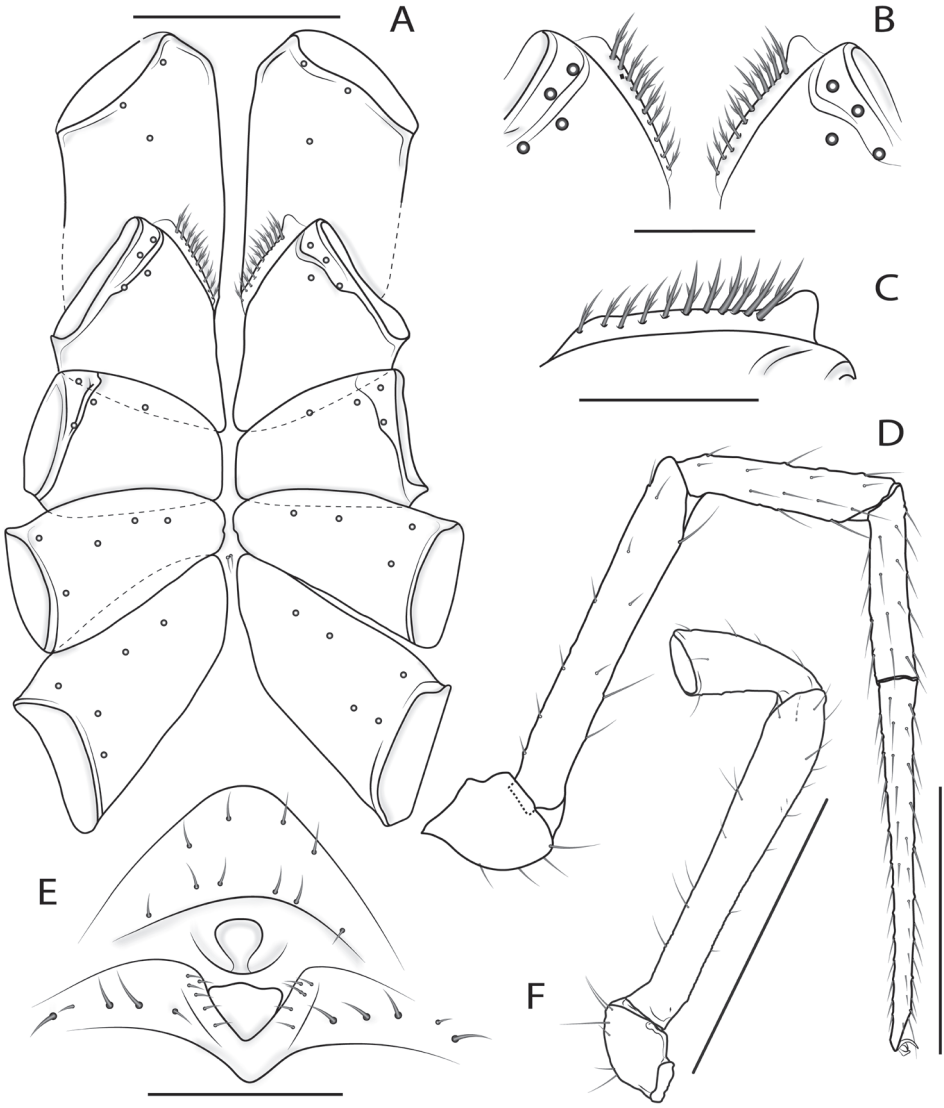

Figure 3. *Selachochthonius naledi* sp. nov. **A** female holotype palp and pedal coxae **B** detail of female holotype coxa I **C** detail of female holotype coxal spines **D** leg I of female holotype **E** male paratype genitalia, showing arrangement of setae **F** right pedipalp of female holotype, dorsal view. Scale bars: 0.3 mm (**A**); 0.1 mm (**B–C**); 0.5 mm (**D**); 0.05 mm (**E**); 1.0 mm (**F**).

the hand on a tiny hump (Fig. 4C–D), adjacent to each other, *eb* proximad to *esb*, *ist* distad to *esb* (about the same distance between both), *eb-esb-ist* located at the base of fixed finger, *it* distad to *est*, *et* distad from *it*. Trichobothrium *st* located at third teeth level, *sb* proximad to *b*, *t* distad to *b*, *b* at the same distance from *t* and *sb* (Fig. 4C–D). Fixed finger almost straight, movable finger slightly bent (Fig. 3A–D). Chelal fixed finger with 32–34 acute, triangular, and widely spaced teeth. Movable finger with

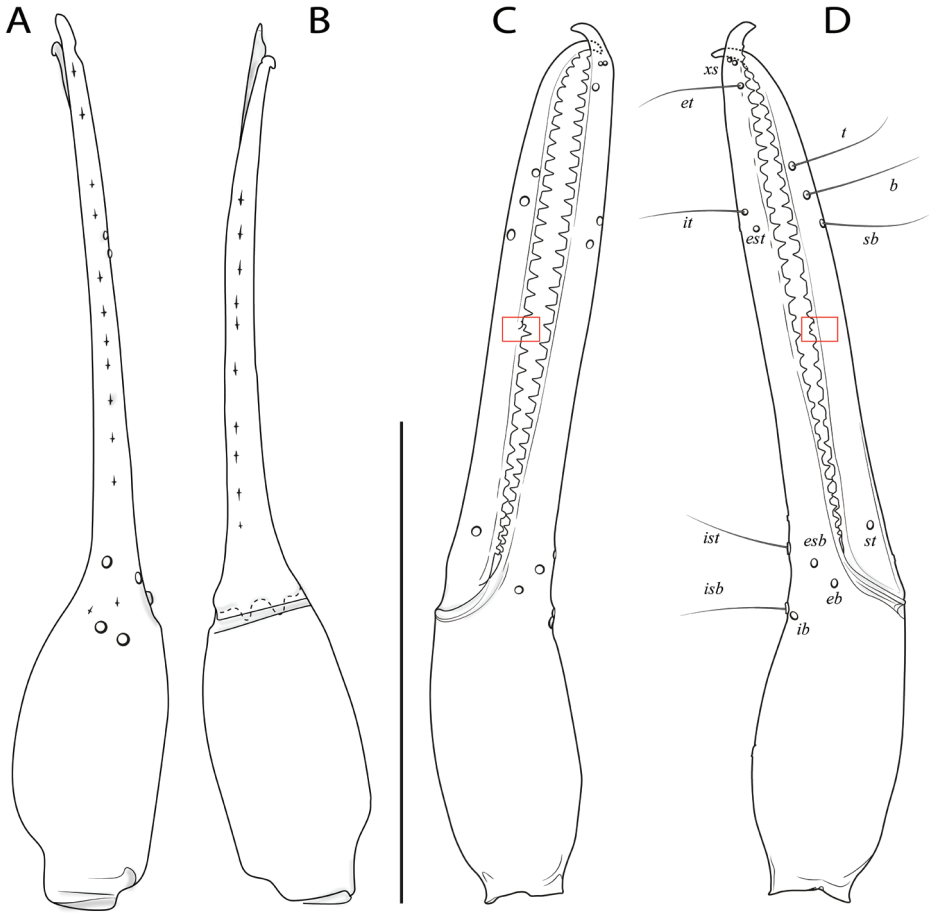

Figure 4. *Selachochthonius naledi* sp. nov., female holotype **A** right pedipalp chela, showing lyrifissures and trichobothrial pattern, dorsal view **B** right pedipalp chela, showing lyrifissures arrangement, ventral view **C** left pedipalp chela, showing teeth morphology, trichobothrial pattern and tubercle location, antiaxial view **D** right pedipalp chela, antiaxial view. See Material and methods for abbreviations. Scale bar: 1.0 mm.

29–31 acute teeth, growing from basal to distal, tubercle present between the 13th and 14th teeth of the female movable finger and between the 10th and 11th teeth of the male movable finger (Fig. 4C–D).

Leg. IV (Fig. 2E). Arolia same length as claws; a tiny protuberance near end of tarsus.

Measurements. (length/width or depth in mm; ratios in parenthesis calculated by using three significant digits): Female holotype and male paratype range. Body length 2.33 [1.86]. Carapace 0.55–0.70/0.52–0.70 (1.0). Palps: trochanter 0.21–0.35/0.15–0.20 (1.4–1.8), femur 0.93–1.22/0.14–0.20 (6.2–6.6), patella 0.38–0.51/0.16–0.21 (2.3–2.4), chela 1.39–1.8/0.30–0.23 (6.1–6.2), movable finger length 0.92–1.14. Leg I: trochanter 0.13–0.23/0.10–0.18 (1.3), femur 0.44–0.67/0.08–0.10 (5.9–6.8), patella 0.29–0.38/0.07–0.09 (4.1–4.4), femur/patella 1.5–1.8, tibia 0.23–0.35/0.05–

Figure 5. Type locality and habitat of *Selachochthonius naledi* sp. nov. **A** Villa Louise cave entrance **B** Villa Louise cave interior general aspect **C** Villa Louise cave interior, showing altered floor **D** Yom Tov cave entrance **E** live female holotype **F** live male paratype.

0.07 (4.3–5.1), tarsus 0.54–0.65/0.05–0.06 (10.7–11.3). Leg IV: Trochanter: 0.24–0.27/0.13–0.17 (1.6–1.8), femur + patella 0.77–1.30/0.19–0.27 (4.1–4.8), tibia 0.50–0.68/0.10–0.12 (5.2–5.4), basitarsus 0.25–0.30/0.07–0.09 (3.4–3.6), telotarsus 0.55–0.73/0.05–0.06 (10.9–12.1).

Habitat and ecological remarks. Despite extensive efforts in search of invertebrates in the caves, only two specimens of *Selachochthonius naledi* sp. nov. were found,

thus indicating their low population density. In both Vila Louise and Yom Tov caves, the specimens were found in the deeper, moist and aphotic areas. The two individuals were collected from under block rocks in the cave floor. Neither specimens exhibited any phototactic behaviour. An interesting behaviour was observed for the individual from Vila Louise cave (female holotype): when gently touched by the brush bristles, the individual responded aggressively, repeatedly grasping the bristles. Even though one of the authors (RLF) has been collecting cave pseudoscorpions for the last 30 years (especially Chthonioidea), this "aggressive" behaviour has never been recorded by him before. Potential prey in both caves include springtails, juvenile crickets and isopods (Styloniscidae).

Discussion

Selachochthonius taxonomy and distribution in South Africa

Species of *Selachochthonius* have been reported from four areas in South Africa (Fig. 1B). *Selachochthonius serratidentatus* was reported from Grootvadersbosch Nature Reserve (Swellendam, Western Cape Province) and Pirie locality (near King Williams Town, Eastern Cape Province). *Selachochthonius heterodentatus* was recorded from the Table Mountain epigean environment, in the vicinity of Wynberg Cave, while *S. cavernicola* was reported only within Wynberg Cave (Table Mountain, Cape Town, Western Cape Province) (Fig. 1A–C). *Selachochthonius naledi* sp. nov. is the first record for the family in Gauteng Province and was found in Yom Tov and Villa Louise caves in the Cradle of Humankind (Chamberlin 1929; Lawrence 1935; Beier 1955, 1964, 1966; Sharratt et al. 2000).

Overall, the taxonomy of *Selachochthonius* has some shortcomings due to lack of detailed morphology on the characters used to differentiate pseudotyrannochthoniid species. The diagnosis of the genus is largely based on the type species *S. serratidentatus*, originally attributed to *Chthonius* by Ellingsen (1912) and subsequently transferred to the new genus *Selachochthonius* by Chamberlin (1929). Beier (1964), provided additional information on various morphological features based on specimens, some of which were from Lesotho. *Selachochthonius cavernicola* presents unique bipinnate coxal spines on coxa I, in fact, the only species within the genus whose description includes drawings of the coxal spines (Lawrence 1935). The new species bears tripinnate spines, which resembles *Pseudotyrannochthonius* and *Centrochthonius*, individually inserted on a single row on the ledge of first pedal coxa (Fig. 3A–C) (Morikawa 1956; Harms and Harvey 2013; Schwarze et al. 2021). The new species herein described is attributed to *Selachochthonius* due to reduced size of the intercoxal tubercle, the smooth aspect of coxal spines and the presence of 18 carapacal setae. As for *S. serratidentatus* and *S. heterodentatus*¸ published descriptions lack detailed information on the morphology of the coxal spines (Ellingsen 1912; Beier 1955, 1964).

The absence of eyes or eyespots — a feature frequently attributed to subterranean-adapted species — is observed in the new species and in *S. cavernicola* (Lawrence

1935). The limited measurements in the original descriptions of *S. cavernicola* and *S. heterodentatus* make comparisons between the length/width ratio of the appendages difficult (Lawrence 1935; Beier 1955) but would be useful to detect if they are somehow elongated – another important troglomorphic trait for pseudoscorpions (Viana & Ferreira 2021). When compared to *S. serratidentatus*, the new species exhibits a slender palpal femur (4.0 times longer than wide in *S. serratidentatus* and 5.9–6.8 times in *S. naledi* sp. nov.) (Lawrence 1935).

Currently, *S. serratidentatus* and *S. heterodentatus* are only found in epigean environments, thus not presenting any subterranean-adapted traits (Ellingsen 1912; Chamberlin 1929, 1962; Beier 1932, 1964), whilst the species encountered inside subterranean environments (*S. cavernicola* and *S. naledi* sp. nov.) exhibit strong troglomorphisms such as depigmented integument and the absence of eyes/eyespots. This supports their status as troglobitic (Lawrence 1935; Chamberlin 1962). However, this classification may be confirmed or disregarded following future surveys aimed at generating additional information on the distribution of epigean species, especially those associated with cave entrances.

Conservation issues

The external area surrounding the caves in the Cradle of Humankind is highly altered, especially by deforestation. The caves' entrances are usually associated with small bush patches surrounded by grass (Fig. 5A–D). There are several farms in the area and a large road network including both paved and unpaved roads. Furthermore, potentially polluting activities take place in the region, such as an asphalt factory located approximately 70 meters from the entrance of Vila Louise Cave. It is important to note that this area is located within what is internationally known as the "Cradle of Humankind" where many hominid fossils were recovered, especially from caves (Berger et al. 2015). Even so, the area is heavily impacted by anthropogenic activities, such as farms and quarries (Fig. 1C).

The caves in the surrounding region are also heavily impacted. For example, the calcite deposits in many of the caves were previously mined. Vila Louise Cave is highly altered due to past removal of calcite. As a result, the cave's conduits and floor were severely changed in part by walls that were built inside the cave (Fig. 5B) and tires that were installed to serve as a staircase in the entrance chamber (Fig. 5C). Even today the cave still receives local visitors with signs of religious use present (e.g. candles and jars). Yom Tov Cave is more preserved, probably due to the difficulties in accessing the deeper sections of the cave that can only be reached via very narrow and vertical passages. Furthermore, there is evidence of a gate that was once installed to prevent local people from accessing the cave.

A major concern related to this species' conservation is the risk of contaminants originating from the farms and small factories in the surrounding area entering the cave systems. Furthermore, it is important to visit other caves in the area to search for additional specimens and determine, with accuracy, the actual distribution of this species.

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RESEARCH ARTICLE

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A new species of *Chaimowiczia* from the karstic Serra do Ramalho plateau, Brazil (Oniscidea, Synocheta, Styloniscidae)

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Abstract

Chaimowiczia belongs to the subfamily Iuiuniscinae, family Styloniscidae, and is currently composed of two troglobitic species: *C. tatus* and *C. uai*, both occurring in Brazil. A new species of Styloniscidae found in Brazil (Bahia state, Serra do Ramalho, Serra Verde cave) was allocated into this genus by the antenna flagellum with three articles; rectangular-shaped lateral pereonites epimera not apically acute and apex of pleopod 2 endopod with an acute tip and an acute or rounded lobe directed outwards. The new species, *C. obybytyra* **sp. nov.**, is here described and rises to 25 the number of troglobitic styloniscidean species known for Brazilian caves.

Keywords

amphibious isopods, Bahia state, Isopoda, troglobite, Neotropics, São Francisco basin

Introduction

Chaimowiczia Cardoso, Bastos-Pereira, Souza & Ferreira, 2021 consists in a recently described genus for the subfamily Iuiuniscinae (Styloniscidae) (Cardoso et al. 2021). The species allocated into the new genus presented the antenna flagellum with three articles; rectangular-shaped lateral pereonites epimera not apically acute and apex of pleopod 2 endopod with an acute tip and an acute or rounded lobe directed outwards (Cardoso et al. 2021).

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Chaimowiczia is currently composed of two species: *C. tatus*, Cardoso, Bastos-Pereira, Souza & Ferreira, 2021 found in Gruta do Padre (Bahia state), and *C. uai*, Cardoso, Bastos-Pereira, Souza & Ferreira, 2021, found in Lapa d'Água do Zezé cave (Minas Gerais state), both in Brazil. Here another species of *Chaimowiczia* is described for a cave in the Bahia state, which consists in a troglobite, as well as most of the styloniscids found in Brazil (Cardoso et al. 2021). Such description rises to 25 the number of troglobitic styloniscidean species known for Brazilian caves (Cardoso et al. 2020a, b, 2021; Campos-Filho et al. 2022).

Materials and methods

Study area

The region of Serra do Ramalho, where Serra Verde cave is located, is one of the most important karstic areas in Brazil (Auler et al. 2001), represented by a huge carbonate plateau with more than 70 km (in the N-S direction) between Corrente river (North) and Carinhanha river (South), in the left bank of the São Francisco River. The carbonate outcrops rise between 550 and 780 m above sea level. The region is inserted in the Caatinga domain (the only Brazilian semiarid biome), with transitional areas to the Cerrado (Brazilian Savanna) (Cole 1960). The local climate is "Aw", according to Köppen's climate classification system, with dry winter and an average annual rainfall of 640 mm^3 (Alvares et al. 2013). The local karst presents a strongly undulating relief, with epigean drainages typically ephemeral, forming deep-steep incisions (da Silva et al. 2019). The area presents more than 180 known caves, some of them among the biggest caves recorded for Brazil, many with more than 5 km in extension (Auler et al. 2001).

Laboratory procedures

The specimens were manually collected with the aid of a hand net and fixed in 70% ethanol. The studied individuals were measured and photographed with a ZEISS Axio ZoomV16 stereomicroscope coupled with an Axio Cam 506 Color camera, dissected and mounted in slides using Hoyer's medium in the Center of Studies on Subterranean Biology of the Federal University of Lavras (**CEBS-UFLA**, Lavras, Brazil). Drawings were made either from photographs or from dissected specimens mounted in slides with the aid of a camera lucida coupled with the microscope Leica DM750. Later the illustrations were prepared in the software GIMP (v. 2.8) (Montesanto 2015, 2016) with a Cintiq Drawing Pad (Wacom). Some specimens were put into an ultrasonic bath (L200 Schuster) to clean the sediment adhered to the cuticle and mounted in stubs for posterior observation of dorsal cuticular structures under the scanning electron microscope Hitachi TM4000. Holotype and paratypes of the new species were deposited in the Subterranean Invertebrate Collection of Lavras (**ISLA-UFLA**) in the Center of Studies on Subterranean Biology of the Federal University of Lavras (CEBS-UFLA, Lavras, Brazil).

Taxonomy

Family Styloniscidae Vandel, 1952
Genus *Chaimowiczia* Cardoso, Bastos-Pereira, Souza & Ferreira, 2021

***Chaimowiczia obybytyra* sp. nov.**
http://zoobank.org/E1EDC0BF-A3B5-48A7-B0D0-873DA3D7EC80

Type species. *Chaimowiczia tatus* Cardoso, Bastos-Pereira, Souza & Ferreira, 2021.

Material examined. ***Holotype*:** Brazil • Male; Bahia state, municipality of Coribe, Serra Verde cave; 13°43'26.03"S, 44°19'24.46"W; 20 Sep. 2021; G.M. Cardoso and R.L. Ferreira leg.; ISLA 95829. ***Paratypes*:** • 4 males; same data as for holotype; ISLA 95830 • 17 females, same data as for holotype; ISLA 95831.

Description. Maximum length: 12 mm. Colorless, eyes absent (Figs 1A, 6C, D). Dorsal surface smooth with sparse scale setae with short triangular base, long sensory sheathed hair and plaques with serrated distal margin (Fig. 1B). Cephalon (Fig. 1C, D) suprantennal line well defined, directed downwards and truncated in middle; round antennal lobes. Body (Figs 1A, 2A) convex, pereonites 1–7 epimera quadrangular, widely separated and outwardly extended, pereonites postero-lateral corners progressively directed backwards; pleon epimera 3–5 well developed, pleonite 5 distal margin as long as telson. Telson (Fig. 1E) distal half subtriangular, depressed with round apex. Antennula (Fig. 2B) with three articles divided by thin suture, covered with thin setae, distal article with one lateral and two apical aesthetascs. Antenna (Figs 1F, 2C) reaches pereonite 2 when extended backwards, fifth article of peduncle longer than flagellum; flagellum with three articles, first article longest. Mandibles as in Fig. 2D, E. Maxillula (Fig. 2F) outer ramus with 5 + 5 teeth, apically entire, and two thick plumose stalks; inner ramus with three slender penicils. Maxilla (Fig. 2G) bilobate, inner lobe wider than outer, covered by setae. Maxilliped (Fig. 2H) basis trapezoidal, distal portion wider than basal; palp apex with tufts of setae; endite shorter than palp, setose, apex with one conic penicil between two strong teeth. Pereopod 1 (Figs 1G, 3A) antennal grooming brush longitudinally on frontal face of carpus and propodus, dactylus with one claw; pereopod 7 with water conducting scale rows. Uropod (Fig. 1E) protopod surpasses distal margin of telson; exopod slightly longer than endopod, inserted at the same level, covered with pectinate scales.

Male. Pereopods 1, 6 and 7 (Figs 1G, 3F, 4A) covered with setae; merus sternal margin with proximal tuft of setae. Genital papilla triangular (Fig. 4B). Pleopod 1 (Fig. 4B) protopod trapezoid, sinuous margin, apex tapering; exopod covered with setae, triangular with sinuous external margin; endopod as long as exopod, flagelliform distal article. Pleopod 2 (Fig. 4C) exopod semi-oval, round distal margin, covered with setae; endopod of two articles, basal article quadrangular, shorter than exopod, distal article stout, apex with acute lobe (perpendicular) directed outwards. Pleopod 3 exopod (Fig. 4D) trapezoid, margin covered with thin setae, ventral face with lobe to hook pleopod 2. Pleopod 4 exopod (Fig. 4E) rhomboid, wider than

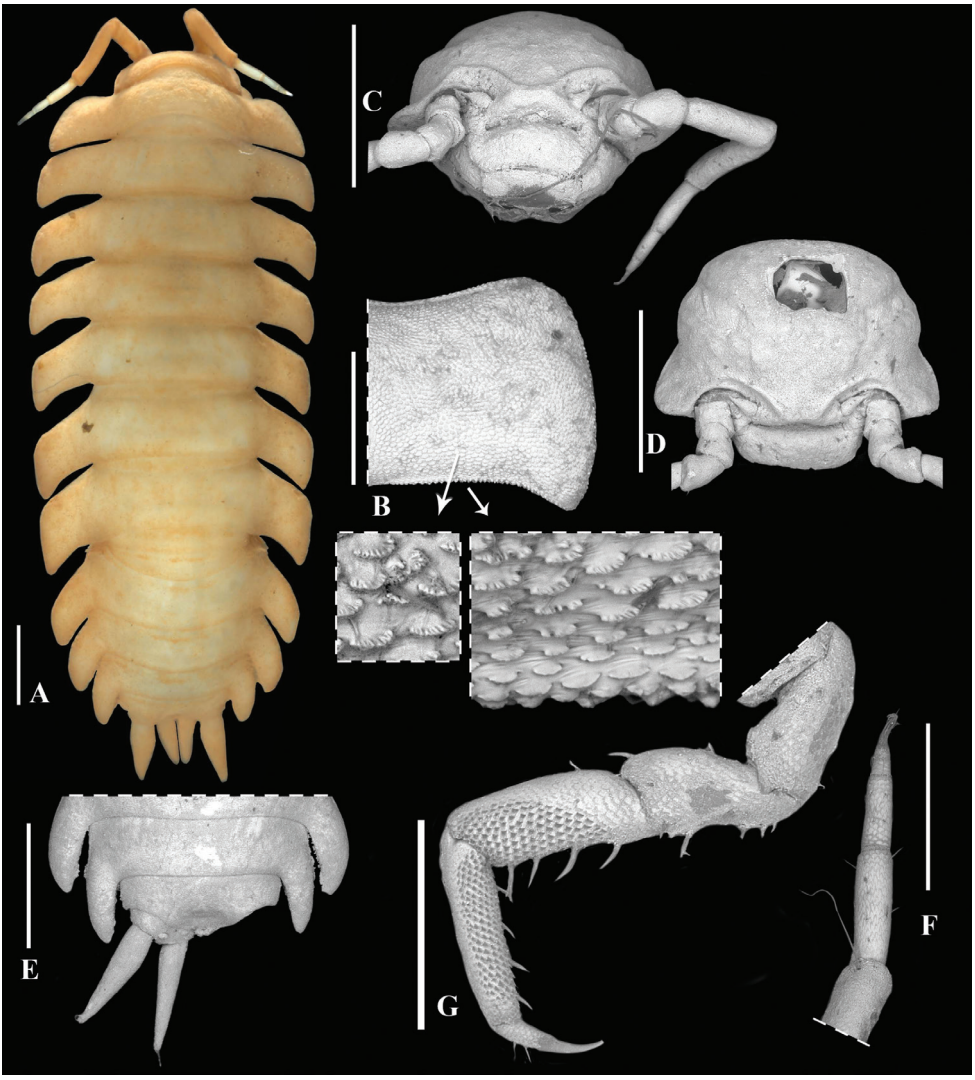

Figure 1. *Chaimowiczia obybytyra* sp. nov. Female paratype **A** habitus dorsal view **B** epimeron 1, dorsal view **C** cephalon, frontal view **D** cephalon, dorsal view **E** pleonites 4 and 5, uropod and telson, dorsal view **F** antennal flagellum **G** pereopod 1. Scale bar: 1mm (**A, C–E**); 500 µm (**B, G, F**).

long, covered with thin setae. Pleopod 5 exopod (Fig. 4F) ovoid, wider than long, covered with thin setae.

Etymology. The epithet "*obybytyra*" was given in reference to the name of the cave Serra Verde (in English, Green Mountain), that in the local Indian language "tupi-guarani" means: oby = green and ybytyra = mountain.

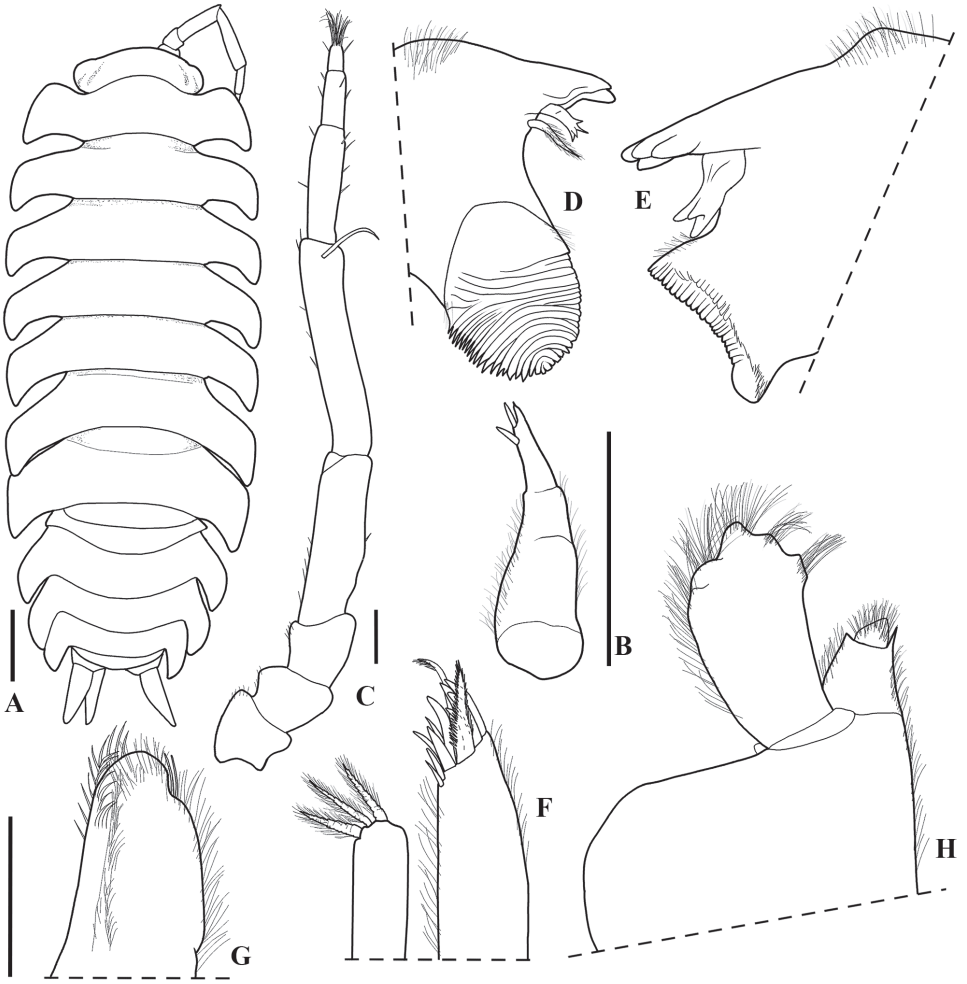

Figure 2. *Chaimowiczia obybytyra* sp. nov. Female paratype **A** habitus, dorsal view. Male paratype **B** antennula **C** antenna **D** right mandible **E** left mandible **F** maxilla **G** maxillula **H** maxilliped. Scale bars: 1 mm (**A**); 0.2 mm (**B–I**).

Discussion

Chaimowiczia obybytyra sp. nov. is larger than the other two species of the genus. The new species is similar to *C. uai* due to the antennal lobes and shape of pereonite 1 epimera, while the similarity with *C. tatus* is in the pleopod 2 endopod apex. A comparison among the morphology of the three species is presented on Table 1.

Chaimowiczia tatus and *C. obybytyra* sp. nov. inhabit caves situated around 63 km far from each other, while *C. uai* represents the southernmost record of the genus until the present (around 143 km far from *C. obybytyra* sp. nov.) (Fig. 5). Besides the distance, geographical barriers exist among the three species, since Corrente River separates the

Table 1. Comparative morphological characters for the three species of *Chaimowiczia*.

Characters	*C. tatus*	*C. uai*	*C. obybytyra* sp. nov.
Body size (mm)	9	8	12
Antennula distal article: number/ position of aesthetascs	two apical	two apical	one lateral, two apical
Antennal lobes	quadrangular	rounded	rounded
Anterior portion of pereonite 1: epimera directed	outward	frontward	frontward
Pleonite 5 posterior margin ___ telson	shorter than distal margin of telson	surpassing distal margin of telson	as long as telson
Uropod endopod ___ exopod	endopod longer than exopod	endopod as long as exopod	endopod slightly shorter than exopod

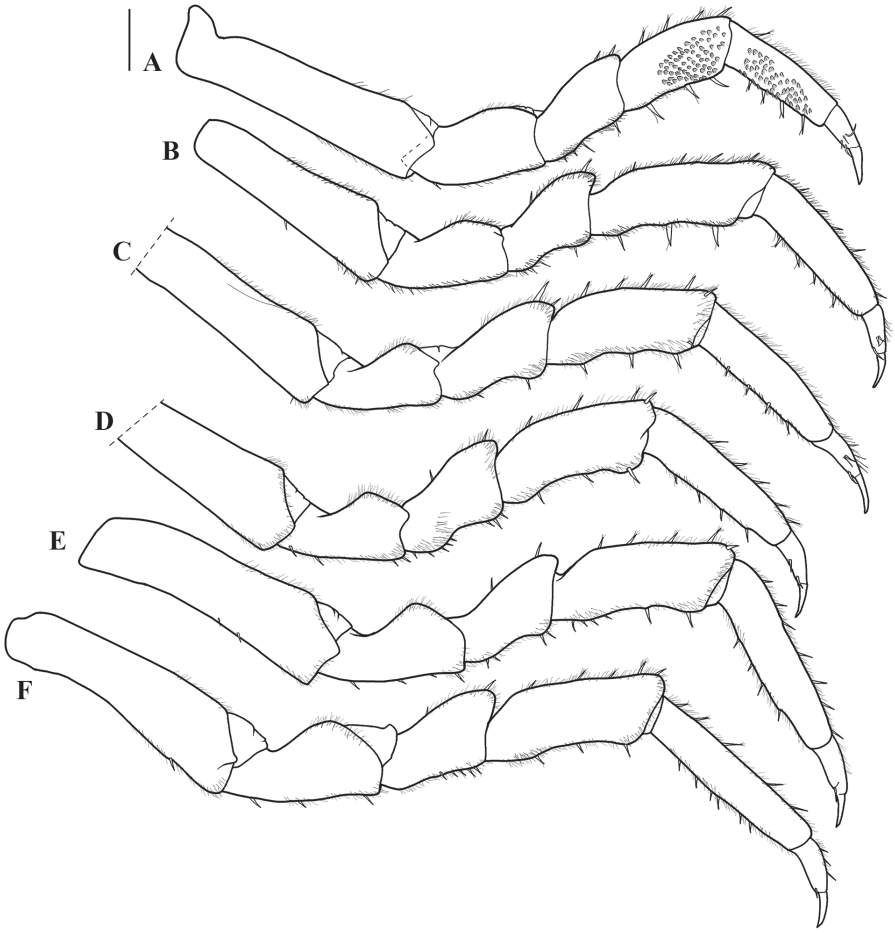

Figure 3. *Chaimowiczia obybytyra* sp. nov. Male paratype **A** pereopod 1 **B** pereopod 2 **C** pereopod 3 **D** pereopod 4 **E** pereopod 5 **F** pereopod 6. Scale bar: 0.2mm.

region of Santana, where *C. tatus* occur, from Serra do Ramalho, where *C. obybytyra* sp. nov. was found. *Chaimowiczia obybytyra* sp. nov. and *C. uai* are also separated by Carinhanha River. These rivers may have represented vicariant events that separated the species along the speciation process within such genus of Styloniscidae in those Brazilian caves.

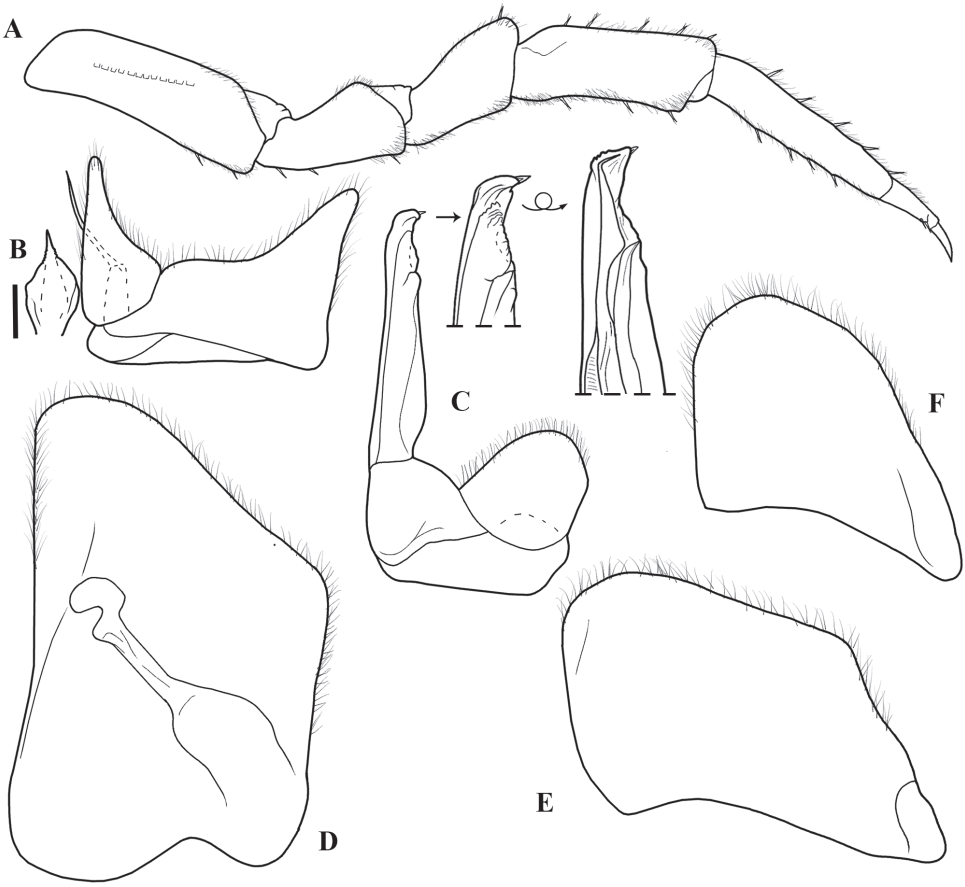

Figure 4. *Chaimowiczia obybytyra* sp. nov. Male paratype **A** pereopod 7 **B** genital papilla and pleopod 1 **C** pleopod 2 **D** pleopod 3 exopod, ventral view **E** pleopod 4 exopod, dorsal view **F** pleopod 5 exopod, dorsal view. Scale bar: 0.2mm.

Habitat and ecological remarks

Specimens of *Chaimowiczia obybytyra* sp. nov. were only observed in the Serra Verde cave. Such cave has at least 1,730 meters of horizontal projection, presenting the main conduit from which secondary passages develop. It is important to note that the cave is still under exploration, and the area where the specimens were collected was not mapped, since it remains flooded along the rainy period. Although the cave presents a wide entrance (Fig. 6A), there is a constriction on the cave conduit (20 meters from the entrance), which is quite long, thus preventing the general public to access the interior of the cave. The conduit that follows this constriction is relatively dry, although there are signs that the runoff water penetrates the cave in rainy periods. Since our team visited the cave during the dry period, the substrates were predominantly dry (near the entrance), however, as entering into the cave, its galleries become progressively wetter, and the conduits in the final portion were quite wet (Fig. 6B). Specimens of

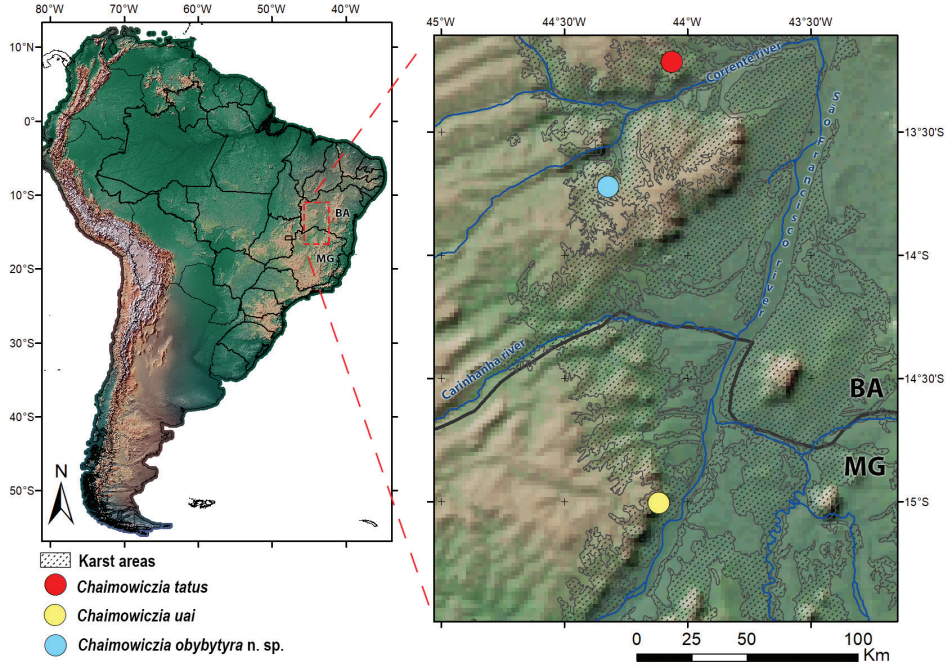

Figure 5. Map with the distribution of *Chaimowiczia* species in the Brazilian states of Bahia and Minas Gerais.

Chaimowiczia obybytyra sp. nov. (Fig. 6C) was found in small ponds in a lower-level conduit, which still maintained these small water collections, even at the height of the drought season. An expedition accomplished by speleologists during the rainy season revealed the inner conduits partially flooded, thus indicating the wide variation in the water table between dry and rainy seasons.

Chaimowiczia obybytyra sp. nov. also exhibits well-developed epimera on the pereonites and pleonites 3–5, as on the other two congeneric species *C. uai* and *C. tatus*. Cardoso et al. (2021) hypothesized that such morphology could be related to a "ghost predation in the past" in a period when the ancestor populations could be under a predator selective pressure. However, this hypothesis still needs further studies in order to be confirmed (or refuted). It is interesting to highlight, however, the co-occurrence of *Chaimowiczia* species with amphipod species of the genus *Spelaeogammarus* (Amphipoda: Artesiidae). All the known species of *Chaimowiczia* share their habitat with such amphipods: *C. uai* co-occur with *S. uai* Bastos-Pereira & Ferreira, 2017; *C. tatus* co-occur with *S. santanensis* Koenemann & Holsinger, 2000; and *C. obybytyra* sp. nov. co-occur with an undescribed species of *Spelaeogammarus* (Fig. 6D) (Bastos-Pereira & Ferreira 2017; Cardoso et al. 2021). Although it is very unlikely that the amphipods could act as predator of adult isopods, it is interesting to consider at least an eventual competition for the scarce food resources occurring inside the caves. Furthermore, while adults of both isopod and amphipod are comparable in size, predation (which was already observed for *Spelaeogammarus* species – RLF personal observations) could

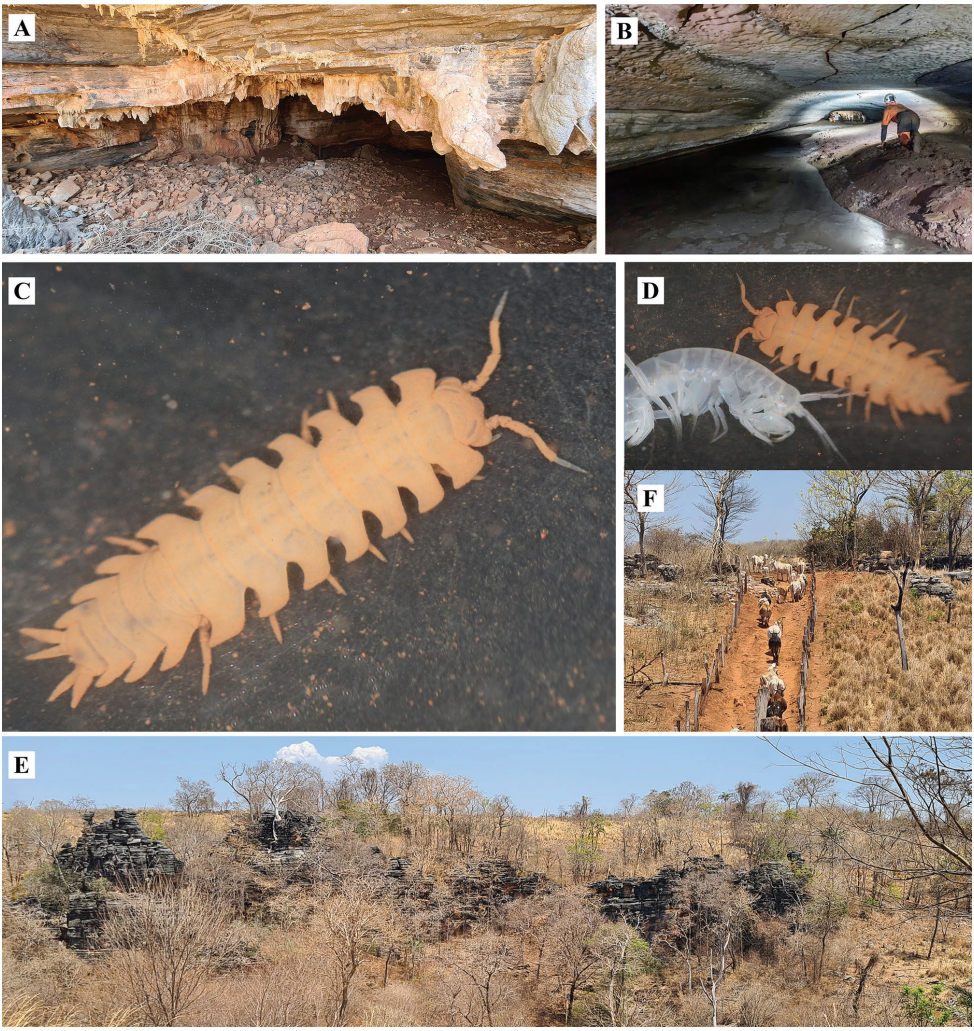

Figure 6. A Serra Verde cave's entrance **B** main conduit in rainy season **C** specimen of *Chaimowiczia obybytyra* sp. nov., *in situ* **D** specimen of *Chaimowiczia obybytyra* sp. nov. and *Spelaeogammarus* sp., *in situ* **E** pastures and livestock in the vicinity of the cave **F** Karst outcrop where the cave is located (Photo by Daniel Menin).

eventually occur between adult amphipods and juvenile isopods, though this is still speculative. In that case, the predation could be also acting currently, not only in the past, as hypothesized by Cardoso et al. (2021).

Two other aspects are also worth mentioning in this context: the first is the fact that all *Chaimowiczia* species have the body surface covered by sediment (which gives the species a brownish color, Fig. 6C). Such sediment adheres to the dorsal modified scales. Hence, living specimens of *Chaimowiczia* present the body surface quite similar in texture to the sediment with which they are associated. This may eventually

represent a texture camouflage, in the case that adult *Spelaeogammarus* sp. feed on juvenile *Chaimowiczia*. The second fact is related to apparent niche segregation between *Chaimowiczia* and *Spelaeogammarus* species. While the former is benthic, frequently found digging the sediment and burying themselves, *Spelaeogammarus* sp. is an active swimming species (RLF personal observation). Again, further studies are highly advisable in order to address such hypothesis, not only based on observations *in situ* but also under laboratory conditions.

Conservation issues

As previously mentioned, the constriction on the conduit close to the entrance prevents residents to access the cave's inner portion. Hence, the cave is quite preserved. However, the external landscape surrounding the cave is severely altered. Only remnants of the original vegetation remain, especially close to the limestone outcrops (Fig. 6E). Most of the pristine forests were removed for pastures. During our visit, cattle were observed in the cave surroundings (Fig. 6F), compacting the exposed soil, which is highly vulnerable to erosional processes. Furthermore, the topography of the external area surrounding the cave entrance (which is located at the bottom of a valley) certainly contributes to the input of external sediments to the cave during rainy periods. Thus, the denudation of external soils can intensify erosive processes and, consequently, increase the sediment input to the cave, silting up microhabitats. It is important to note that other cave-restricted species were observed in this cave, as the undescribed species of *Spelaeogammarus*, millipedes, springtails, among others, which shows the biological relevance of this cave. Accordingly, it is highly recommendable that the cave surrounding be protected, especially by the reforestation of the immediate external landscape, to protect the cave and consequently the unique species it presents.

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RESEARCH ARTICLE

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Seasonal abundance and spatio-temporal distribution of the troglophylic harvestman *Ischyropsalis ravasinii* (Arachnida, Opiliones, Ischyropsalididae) in the Buso del Valon ice cave, Eastern Italian Prealps

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Abstract

We explore the population of the troglophilic harvestman *Ischyropsalis ravasinii* inhabiting the Buso del Valon ice cave located in the Italian Prealps. Spatial and temporal distributions of the specimens are investigated in relation to the variation of environmental abiotic conditions in the cave, such as the seasonal temperature and substrate surface typology. Our results show that *I. ravasinii* is distributed unevenly in the cave, most of individuals being present in the scree-covered section of the cave with superficial activities limited to the warm seasons only. In addition, our data suggests that the presence of a thick layer of rocky debris, together with high humidity and cold temperatures, are important limiting factors for the species. Seven additional species of harvestman are recorded in the cave, including the congeneric troglophilic species *Ischyropsalis strandi*. This is the first known record of these two troglophilic *Ischyropsalis* species coexisting within the same cave. An updated map of the distribution of *I. ravasinii* and *I. strandi* in the Italian Prealps is provided.

Keywords

Age classes, global warming, Lessinia Mountains, Northern Italy, seasonality, subterranean environment

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Introduction

Harvestmen (Arachnida: Opiliones) are one of the largest orders within the class Arachnida, numbering 65 families and 6637 species (Blick and Harvey 2011; Kury et al. 2020). Harvestmen show highly diverse morphology and biology, allowing them to successfully colonize a large number of habitats including terrestrial and subterranean habitats. The genus *Ischyropsalis* C.L. Koch, 1839 (Family Ischyropsalididae Simon, 1879) contains some of most iconic European harvestmen, which are easily recognizable by their relatively large body size, massive, prominent chelicerae and dark coloration. Numbering 22 species, all geographically limited to Europe, *Ischyropsalis* species are characterized by a high level of endemism. They are often restricted to a single mountain chain (Schönhofer 2013; Schönhofer et al. 2015). They frequently show frigophilic and hygrophilic habits, having marked preferences for microhabitats with low temperature and constantly high humidity (Martens 1969; Schönhofer et al. 2015). Recent studies also suggest a direct relationship between the current distribution of Alpine *Ischyropsalis* species and the Pleistocene glaciations (Mammola et al. 2019). Although in high mountains these arachnids can be found in open or shallow humid habitats, such as scree and mossy landscapes, at lower altitudes they inhabit caves and other subterranean habitats. Thus, several *Ischyropsalis* species display an affinity for hypogean habitats and different degrees of adaptations to the subterranean life, including numerous obligate cave-dwellers (Marcellino 1982; Schönhofer et al. 2015). Despite their relevance as part of the European cave-dwelling fauna, the ecology of this genus has been scarcely explored (Martens 1969). To date, only six species have been partially analyzed in terms of ecology and life cycle, and most of the data rely on old studies carried out over 50 years ago (e.g. *I. luteipes* Simon, 1872 and *I. pyrenaea* Simon, 1872 see Juberthie 1961; *I. strandi* Kratochvil, 1936 see Juberthie 1963; *I. kollari* C.L. Koch, 1839 see Martens 1969; *I. dentipalpis* Canestrini, 1872 and *I. lithoclasica* Schönhofer & Martens, 2010 see Schönhofer and Martens 2010). For most known cave-dwelling *Ischyropsalis* no updated information concerning their life cycle, seasonality, micro-habitat preference or even area of distribution are available.

In caves, where the environmental conditions remain rather constant along the year (Badino 2010), abiotic factors represent a critical element to define the structure and composition of the subterranean communities (Howarth 1980). Local variations may deeply affect the spatial and temporal distribution of the cave-dwelling arthropods (Latella et al. 2008). In particular, frigophilic cave species have adapted to living in cold subterranean habitats characterized by low temperature and the presence of an ice or snow layer throughout the year, albeit with some seasonal variation (Iepure 2018).

Located in the Italian Prealps (Fig. 1B) at relatively low altitude, the Buso del Valon ice cave (cadaster number: 438 V/VR) has a permanent internal ice body and superficial snow (Zorzin et al. 2015). The cave hosts a well-defined assemblage of arthropods adapted to cold environments including three species of the wingless limoniid crane flies of the genus *Chionea* Dalman, 1816 (Avesani and Latella 2016; Latella et al. 2019). It also shelters a large population of the harvestman *I. ravasinii*

Hadži, 1942 (Fig. 1D), a troglophilic species endemic to the Venetian Prealps in North-East Italy. Due to its unique features, the Buso del Valon ice cave represents an ideal natural laboratory to explore the phenology and microhabitat preference of frigophilic subterranean species (Avesani and Latella 2016). With this study, we aim to complete our knowledge of the life cycle and spatio-temporal distribution of *I. ravasinii* living in cold subterranean environments in relation to the cave substratus and seasonal changes. We further plan to use data herein collected as a starting point for long-lasting studies aiming to monitor the effects of climate change on the frigophilic cave fauna and its resilience to changes in micro-habitat conditions (see e.g. Howarth, 2021).

Materials and methods

Area of study

The Buso del Valon ice cave (Fig. 1A) is located at ~1700 m a.s.l. in the Lessini Mountains of the Venetian Pre-Alps in Northern Italy (Veneto Region, Province of Verona, 45°41'32.26"N, 11°0.6"11.10"E) (Fig. 1B). The mountain chain forms a trapezium-shaped massif dominated by Mesozoic and Cenozoic limestones. These sedimentary rocks are interspersed by Cenozoic volcanic rocks and Eocenic limestone outcrops (Sauro 1973). The cave opens with a large shaft approximately 30 m in diameter and 50 m deep. It shows a vertical E-W course reaching nearly 70 m of depth at its deepest point (Fig. 1A, C). The Buso del Valon ice cave is one of the few karstic cavities of the Veneto Region with permanent cold internal temperatures ranging from -8 °C to 7 °C (Fig. 2A) and hosting a permanent ice body fed by seasonal snowfalls through the entrance. The ice inside the cave has been retreating in recent years, probably due to climate change (Latella et al. 2019).

Specimens sampling

Field collections were carried out in the cave for approximately two and half years, from July 2014 to December 2016 resulting in a consecutive temporal series of 30 months. We selected five sampling stations inside the cave (ST1–4 and DPS), located in three ecologically different areas of the cave: one at the entrance shaft base (ST1), two on the scree near the border of the internal ice layer (ST2, DPS), two near the bottom (ST3 and ST4) (Fig. 1C). Each selected area was characterized by specific combination of abiotic factors (e.g. albedo, typology and thickness of substrate; see Table 1). Four stations (ST1–4) were sampled using standard pitfall traps consisting of a glass cup with an open diameter of 10 cm filled with propylene glycol. A deep scree trap (DPS, Fig. 1E) was set approximately one meter deep inside the scree for monitoring possible seasonal vertical movement of the harvestman specimens among the debris. The DPS trap consisted of a 90 cm long PVC pipe with an inside diameter of 11 cm and several small holes (5–7 mm in diameter) drilled along its surface (see López and Oromí 2010). A 10 cm diameter plastic cup filled with propylene glycol was placed

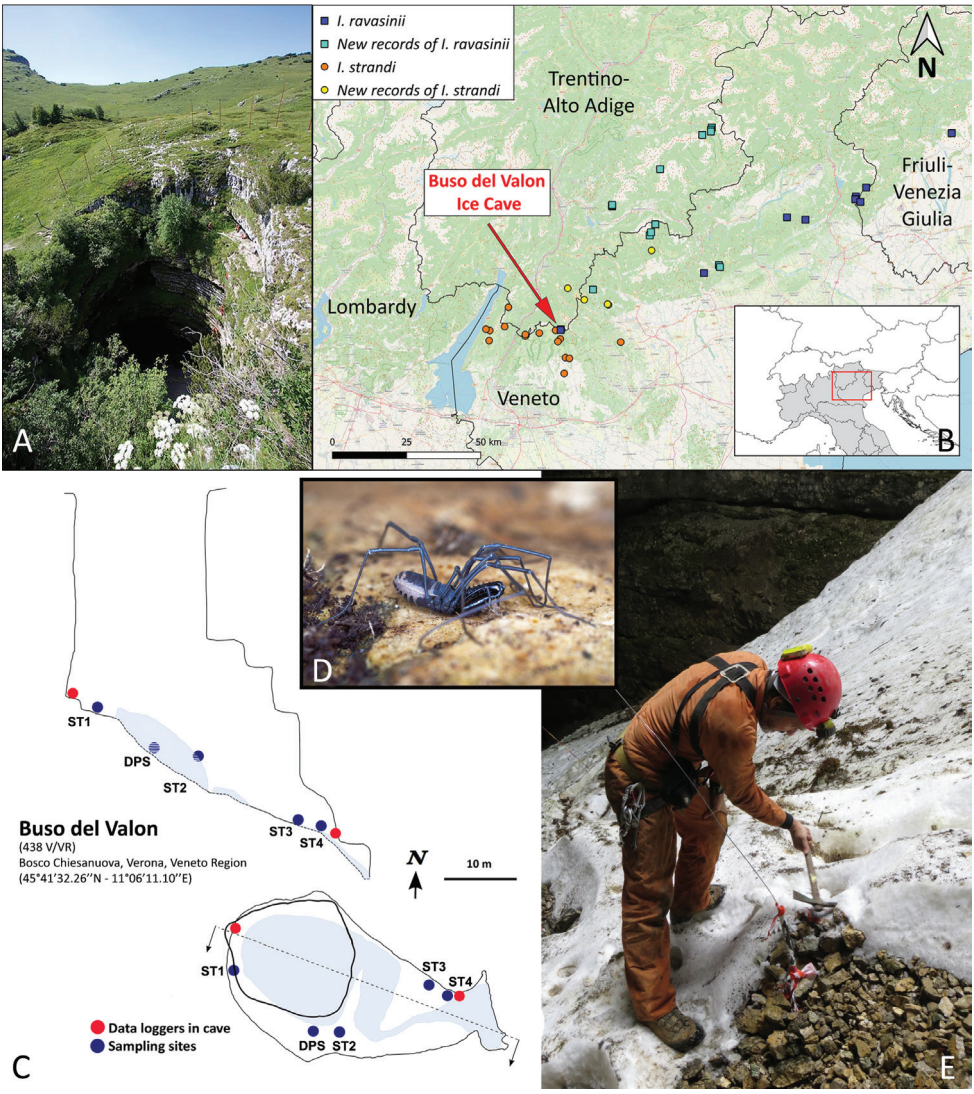

Figure 1. Location and outlines of the study area **A** entrance of the Buso del Valon ice cave **B** updated distribution of *Ischyropsalis ravasinii* and *I. strandi*: the position of the cave is highlighted by an arrow **C** transversal and horizontal sections of the Buso del Valon ice cave (modified from Zorzin et al. 2015), with the locations of the selected stations and dataloggers used in the study. The extension of the permanent ice and snow coverage inside the cave is illustrated in light blue. The external station (ST5) is not shown **D** adult male *Ischyropsalis ravasinii* **E** replacement of the deep scree trap inside the scree. Abbreviations: DPS = sampling station with deep scree trap, ST1–4 = sampling stations 1–4 with pitfall traps.

at the bottom of the pipe to collect the samples. An additional pitfall trap (ST5) was installed outside the cave near its external border acting as a control station. A bait consisting of a piece of blue mould cheese in a plastic vial was added in each trap to attract the cave-dwelling arthropods. The collected specimens were fixed in 75% ethanol for

Table 1. Position and abiotic factors of the stations used in this study. For the exact position of each sampling station see Fig. 1C.

Station	Type of trap	Position	Albedo	Surface typology	Ice/snow coverage
ST1	Superficial pitfall trap	Base of the shaft	Low	Large stones, clay and moss	No
ST2	Superficial pitfall trap	Middle cave section	Low	Thick scree	Yes-seasonal
DPS	Deep scree trap	Middle cave section	Absent	Thick scree	Yes-seasonal
ST3	Superficial pitfall trap	Bottom of the cave	Very low	Large stones and clay	No
ST4	Superficial pitfall trap	Bottom of the cave	Very low	Fissured rock	Yes-seasonal
ST5	Superficial pitfall trap	Outside of the cave	Strong	Meadow soil	No

morphological study. All specimens used in this study are preserved in the collections of the Museo di Storia Naturale of Verona, Italy.

Due to the varying albedo and temperature along the year, the permanent ice and snow layer inside the Buso del Valon ice cave shows seasonal variation in spatial coverage and thickness. Following this feature, the sampling time and consequent analysis of the data was divided into two time-frames of six months each: from mid-June to mid-December and from mid-December to mid-June. Thus, each trap was set in place for a period of 6 months before being emptied and refreshed. These periods roughly correspond to the warm and cold seasons of the year inside the cave, namely the periods of minimum and maximum temperature (Fig. 2A) and extension of the snow and ice coverage inside the cave. Two Tinytag Plus data loggers (-30 °C to +50 °C) were positioned inside the cave for a period of two years, one at the base of the shaft and one in the lower part of the cave (Fig. 1C), to record the temperature changes during the two seasons in different parts of the study area.

Stages identification and population demography

Identification of adults at species level was carried out under a stereomicroscope (Bresser Advance ICD 10-160x) according to Martens (1978). Juveniles of *I. ravasinii* were distinguished from juveniles of other species (e.g. *I. strandi*) based on the presence of eye pigmentation and length of chelicerae basal article. Previous studies have shown that the life cycle of *Ischyropsalis* species is divided into six growing instars before the adult form (Juberthie 1961; Martens 1969). Based on this information, we classified each collected specimen into one of the seven putative stages (instars IN 1–6 and adults AD) based on the length of their cephalothorax, chelicerae and cheliceral spines. These characters are considered discriminant among different instars and thus indicative of the growing stages (Martens 1969).

Adults and instar richness within each trap were calculated summing the number of individuals of *I. ravasinii* collected. Nevertheless, each trap may show unique results due to the different types of traps used (pitfalls and DPS) and slightly different collecting timeframes in different years (see Table 2). To avoid sampling bias and to make the data more directly comparable, for each trap we also defined a trapping rate (TR, *sensu* Vater 2011). Therefore, the number of specimens collected was converted

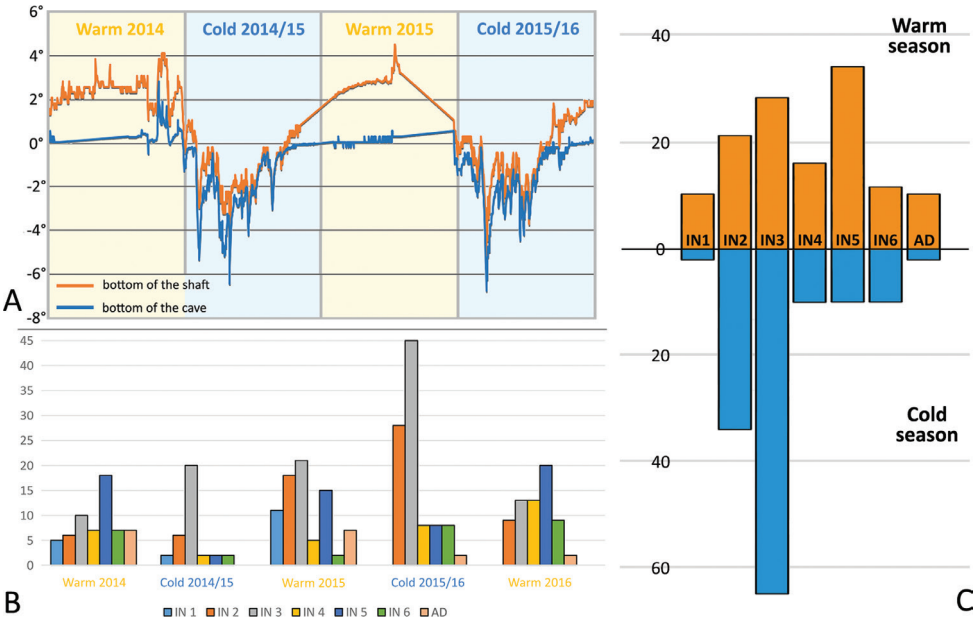

Figure 2. A temperatures recorded over two years in the Buso del Valon ice cave, for the detailed position of the dataloggers see Fig. 1A **B** seasonal instars and adult abundance collected during each separate trapping period **C** number of individuals of different growth stages collected during the warm and cold seasons. Abbreviations: AD = adults, IN 1–6 = instars 1–6.

using the following formula: TR = n° individuals/n° of days of trap activity. Statistical analyses were carried out and graphs were plotted using PAST4 and EXCEL software. The map with the updated distribution of *I. ravasinii* and *I. strandi* was constructed using QGIS version 3.4 including known records from literature and new records collected by the first author.

Captive breeding

In order to obtain supplementary information on the life cycle of *I. ravasinii*, additional specimens were collected from the artificial tunnel Galleria Vittorio Emanuele III located in the Grappa Massif, Venetian Pre-Alps. Three adults and one juvenile belonging to the 5th instar were collected in March 2020 and raised in controlled conditions for approximately 14 months. Specimens were kept in plastic boxes (size 15 × 8 × 10 cm for adults and late instars and 5 × 5 × 3 cm for the early instars) with small stones and wood sticks and a layer of peat on the bottom. The boxes were stored in a fridge with a controlled temperature of 6–8 °C. To maintain constant moisture the boxes were frequently sprayed with nebulized water. Harvestmen were fed using collembola, small flies or crickets. Hatchlings were raised until reaching the 4th instar. Adults and the juvenile collected in the field were raised until the end of their life cycle.

Table 2. List of harvestman species collected in the Buso del Valon ice cave and related stations, including numbers of individuals. Abbreviations: AD = adults, DPS = sampling station with deep scree trap, IN 1–6 = instars 1–6, ST1–5 = sampling stations 1–5 with pitfall traps.

Species	Collection period	Station	N° of specimens	Instar	Season
Ischyropsalis ravasinii	25.IX.2014–14.XII.2014	ST2	1	IN 1	Warm season
Ischyropsalis ravasinii	25.IX.2014–14.XII.2014	ST2	3	IN 1	Warm season
Ischyropsalis ravasinii	25.IX.2014–14.XII.2014	ST2	1	IN 1	Warm season
Ischyropsalis ravasinii	25.IX.2014–14.XII.2014	ST2	2	IN 2	Warm season
Ischyropsalis ravasinii	25.IX.2014–14.XII.2014	ST2	4	IN 2	Warm season
Ischyropsalis ravasinii	25.IX.2014–14.XII.2014	ST2	10	IN 3	Warm season
Ischyropsalis ravasinii	25.IX.2014–14.XII.2014	ST2	6	IN 4	Warm season
Ischyropsalis ravasinii	25.IX.2014–14.XII.2014	ST2	1	IN 4	Warm season
Ischyropsalis ravasinii	25.IX.2014–14.XII.2014	ST2	14	IN 5	Warm season
Ischyropsalis ravasinii	25.IX.2014–14.XII.2014	ST2	3	IN 5	Warm season
Ischyropsalis ravasinii	25.IX.2014–14.XII.2014	ST2	1	IN 5	Warm season
Ischyropsalis ravasinii	25.IX.2014–14.XII.2014	ST2	7	IN 6	Warm season
Ischyropsalis ravasinii	25.IX.2014–14.XII.2014	ST2	6	AD (2♂, 4♀)	Warm season
Ischyropsalis ravasinii	25.IX.2014–14.XII.2014	ST2	1	AD (1♀)	Warm season
Ischyropsalis ravasinii	14.XII.2014–27.VI.2015	DPS	2	IN 1	Cold season
Ischyropsalis ravasinii	14.XII.2014–27.VI.2015	DPS	6	IN 2	Cold season
Ischyropsalis ravasinii	14.XII.2014–27.VI.2015	DPS	20	IN 3	Cold season
Ischyropsalis ravasinii	14.XII.2014–27.VI.2015	DPS	2	IN 4	Cold season
Ischyropsalis ravasinii	14.XII.2014–27.VI.2015	DPS	2	IN 5	Cold season
Ischyropsalis ravasinii	14.XII.2014–27.VI.2015	DPS	2	IN 6	Cold season
Ischyropsalis ravasinii	27.VI.2015–6.XII.2015	ST1	1	IN 5	Warm season
Ischyropsalis ravasinii	27.VI.2015–6.XII.2015	ST2	14	IN 5	Warm season
Ischyropsalis ravasinii	27.VI.2015–6.XII.2015	ST2	2	IN 6	Warm season
Ischyropsalis ravasinii	27.VI.2015–6.XII.2015	DPS	11	IN 1	Warm season
Ischyropsalis ravasinii	27.VI.2015–6.XII.2015	DPS	18	IN 2	Warm season
Ischyropsalis ravasinii	27.VI.2015–6.XII.2015	DPS	20	IN 3	Warm season
Ischyropsalis ravasinii	27.VI.2015–6.XII.2015	DPS	5	IN 4	Warm season
Ischyropsalis ravasinii	27.VI.2015–6.XII.2015	DPS	7	AD (3♂, 4♀)	Warm season
Ischyropsalis ravasinii	27.VI.2015–6.XII.2015	ST3	1	IN 3	Warm season
Ischyropsalis ravasinii	27.VI.2016–8.XII.2016	ST1	1	IN 4	Warm season
Ischyropsalis ravasinii	27.VI.2016–8.XII.2016	ST2	9	IN 2	Warm season
Ischyropsalis ravasinii	27.VI.2016–8.XII.2016	ST2	13	IN 3	Warm season
Ischyropsalis ravasinii	27.VI.2016–8.XII.2016	ST2	9	IN 4	Warm season
Ischyropsalis ravasinii	27.VI.2016–8.XII.2016	ST2	18	IN 5	Warm season
Ischyropsalis ravasinii	27.VI.2016–8.XII.2016	ST2	8	IN 6	Warm season
Ischyropsalis ravasinii	27.VI.2016–8.XII.2016	ST2	2	AD (2♀)	Warm season
Ischyropsalis ravasinii	27.VI.2016–8.XII.2016	ST3	3	IN 4	Warm season
Ischyropsalis ravasinii	27.VI.2016–8.XII.2016	ST3	2	IN 5	Warm season
Ischyropsalis ravasinii	27.VI.2016–8.XII.2016	ST3	1	IN 6	Warm season
Ischyropsalis ravasinii	6.XII.2015–27.VI.2016	ST1	2	IN 3	Cold season
Ischyropsalis ravasinii	6.XII.2015–27.VI.2016	ST2	1	IN 5	Cold season
Ischyropsalis ravasinii	6.XII.2015–27.VI.2016	DPS	28	IN 2	Cold season
Ischyropsalis ravasinii	6.XII.2015–27.VI.2016	DPS	42	IN 3	Cold season
Ischyropsalis ravasinii	6.XII.2015–27.VI.2016	DPS	8	IN 4	Cold season
Ischyropsalis ravasinii	6.XII.2015–27.VI.2016	DPS	6	IN 5	Cold season
Ischyropsalis ravasinii	6.XII.2015–27.VI.2016	DPS	5	IN 6	Cold season

Species	Collection period	Station	N° of specimens	Instar	Season
Ischyropsalis ravasinii	6.XII.2015–27.VI.2016	DPS	2	AD (1♂, 1♀)	Cold season
Ischyropsalis ravasinii	6.XII.2015–27.VI.2016	ST4	1	IN 3	Cold season
Ischyropsalis ravasinii	6.XII.2015–27.VI.2016	ST4	1	IN 5	Cold season
Ischyropsalis ravasinii	6.XII.2015–27.VI.2016	ST4	3	IN 6	Cold season
Gyas annulatus	6.XII.2015–27.VI.2016	ST4	1	1 juv.	Cold season
Gyas annulatus	25.IX.2014–14.XII.2014	ST2	1	1 juv.	Warm season
Gyas annulatus	27.VI.2015–6.XII.2015	ST3	1	1 juv.	Warm season
Gyas annulatus	27.VI.2015–6.XII.2015	ST2	1	1 juv.	Warm season
Histricostoma dentipalpe	27.VI.2016–8.XII.2016	ST1	1	AD (1 ♀)	Warm season
Histricostoma dentipalpe	27.VI.2016–8.XII.2016	ST3	2	AD (1♂, 1♀)	Warm season
Ischyropsalis strandi	27.VI.2015–6.XII.2015	ST2	2	AD (2♀)	Warm season
Ischyropsalis strandi	25.IX.2014–14.XII.2014	ST2	1	IN 2	Warm season
Ischyropsalis strandi	27.VI.2015–6.XII.2015	DPS	1	IN 1	Warm season
Lacinius horridus	27.VI.2015–6.XII.2015	ST5 (external)	1	AD (1 ♀)	Warm season
Lophopilio palpinalis	27.VI.2015–6.XII.2015	ST5 (external)	15	AD (7♂, 8♀)	Warm season
Lophopilio palpinalis	27.VI.2016–8.XII.2016	ST1	1	AD (1 ♀)	Warm season
Lophopilio palpinalis	27.VI.2016–8.XII.2016	ST3	2	AD (1♂, 1♀)	Warm season
Mitopus morio	27.VI.2015–6.XII.2015	ST5 (external)	2	AD (1♂, 1♀)	Warm season
Mitostoma sp.	6.XII.2015–27.VI.2016	DPS	1	1 juv.	Cold season
Nemastoma sp.	27.VI.2016–8.XII.2016	ST2	1	AD (1 ♀)	Warm season
Rilaena triangularis	6.XII.2015–27.VI.2016	ST2	2	AD (2♀)	Cold season

Results

Stages composition and seasonal abundance

A total of 338 specimens of *I. ravasinii* were collected inside the cave during the study period (Table 2). Among them, 18 were adults (6 males, 12 females, 5.3% of the total) and 320 were juveniles (94.7%). Juveniles belonged to the following instars: IN 1=18, IN 2=67, IN 3=110, IN 4=35, IN 5=63, IN 6=28 (Fig. 3A). No specimens of *I. ravasinii* were found in the trap located outside the cave area. Instars and adults abundance in warm and cold periods are illustrated in Fig. 2B, C. During the warm seasons, when the extension of the internal ice layer was at its minimum, a total of 205 specimens were sampled. They were partitioned into the seven stages as follows: IN 1=16, IN 2=33, IN 3=44, IN 4=25, IN 5=53, IN 6=18, AD=16 (Fig. 3A). Juveniles represented the majority of the collected specimens (92.1%), in particular the first three instars (~40% of the total) and especially the IN 5 which alone included ¼ of all the samples. Male/female sex ratio in this season was 5:11. During the cold seasons, in the periods of maximum ice extension,133 specimens were collected: IN 1=2, IN 2=34, IN 3=65, IN 4=10, IN 5=10, IN 6=10, AD=2 (Fig. 3A). Again, the majority of specimens were juveniles (98.5%), in particular those belonging to the early three instars (83.2%). The male/female sex ratio was 1:1.

Spatio-temporal distribution

Most specimens (95% of the total samples) were collected in the middle section of the cave, characterized by a thick layer of rocky debris. Samples were collected both on the surface (ST2: ~40.2%) and in the deep layers (DPS: 55%). All other stations collected a much smaller number of specimens, between 1.2% and 2.1% of the total samples (Fig. 3B).

Similar results were obtained considering the collections occurred only in the warm or the cold seasons. During the warm seasons (Fig.3C) the stations located in the scree showed the highest trapping rate both on the surface and in the deep layers (ST2 TR=0.994, DPS TR=0.449). Few specimens were collected in the other stations, near the entrance (ST1 TR=0.014) or at the bottom of the cave (ST3 TR=0.051; ST4 TR=0.00). During the cold season, all individuals were gathered in the deep layers of the scree, the deep scree trap showing the highest trapping rate (DPS TR=0.626). In contrast, only a few individuals were found on the surface, all the surface pitfall traps showing low trapping rates including in the scree (ST1 TR=0.010; ST2 TR=0.005; ST3 TR=0.00; ST4 TR=0.025) (Fig.3C).

Life cycle in captivity

Eggs were laid in captivity between late April and June 2020 always in the most humid part of the breeding boxes where several condensation drops were present. Each egg cluster contained between 10 to 20 eggs. Egg development, from deposition to hatching, required about 100 days until middle-late August. Only approximately 50% of the eggs hatched. Hatchlings needed about 11 months to reach the 4th instar, each growing stage lasting between one to three months. The juvenile of the 5th instar reached adulthood approximately three months later, in June 2020 and survived as adult for nearly one more year until May 2021. The whole life cycle is estimated to last approximately two years.

Additional notes on the opiliofauna of the Buso del Valon ice cave

In addition to *I. ravasinii*, the congeneric species *I. strandi* Kratochvil, 1936 was sampled in the study area. Only four specimens of *I. strandi* were collected during the two and half years of sampling: two adult females and two juveniles belonging to the 1st and 2nd instars, respectively (Table 2). All the specimens were found in the scree area (ST2 and DPS) during the warm seasons. The small number of individuals did not allow statistical evaluation. An updated distribution of these two *Ischyropsalis* species in the Italian Prealps is illustrated in Fig. 1B. Six additional species of harvestmen belonging to two different families and six different genera were also sampled inside the Buso del Valon cave: Fam. Nemastomidae: *Histricostoma dentipalpe* (Ausserer, 1867) (3 spec.); *Mitostoma* sp. (1 spec.); *Nemastoma* sp. (1 spec.); Fam. Phalangiidae: *Gyas annulatus* (Olivier, 1791) (4 spec.); *Lophopilio palpinalis* (Herbst, 1799) (18 spec.); *Rilaena triangularis* (Herbst, 1799) (2 spec.) (see Table 2).

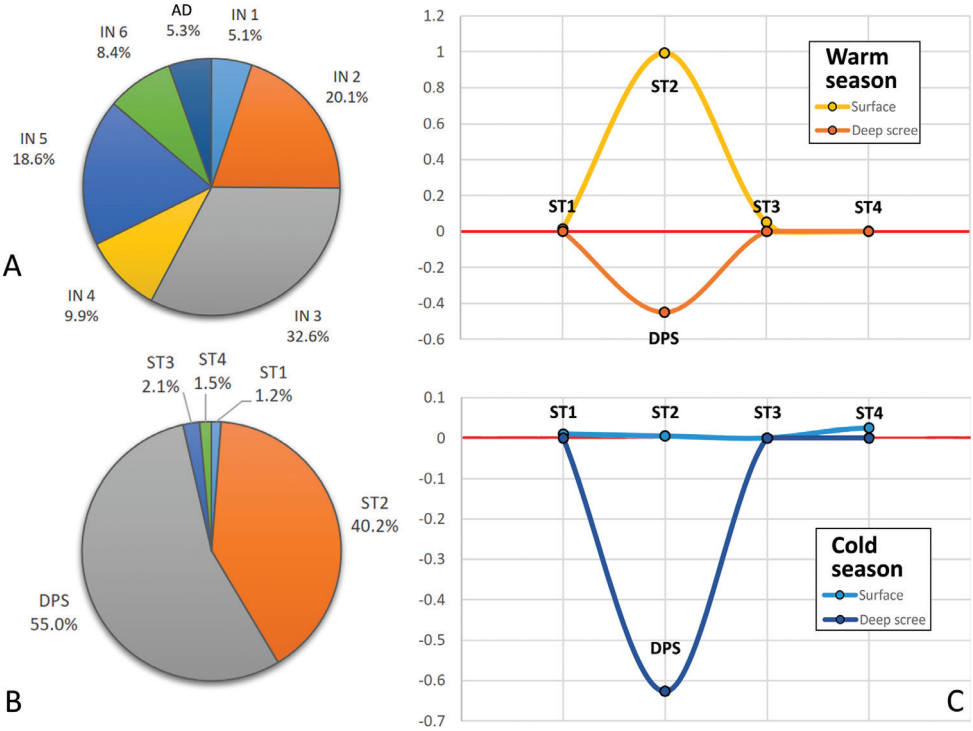

Figure 3. A percentage of instars and adult relative abundances **B** percentage of sampling relative abundances by station **C** comparison of the trapping rate (TR) of each station during the warm and cold seasons. Abbreviations: AD = adults, DPS = sampling station with deep scree trap, IN 1–6 = instars 1–6, ST1–4 = sampling stations 1–4 with pitfall traps.

Discussion

Among the harvestman fauna inhabiting the Buso del Valon ice cave, two coexisting species belonging to the genus *Ischyropsalis* were collected*: I. ravasinii* and *I. strandi*. Both the species belong to the Alpine clade *sensu* Schönhofer et al. 2015, and show a reduced distribution along the Venetian Prealps. *Ischyropsalis strandi* is endemic to the Lessini and Baldo mountains, while *I. ravasinii* extends its distribution to the East toward the Cansiglio plateau. Thus, the Buso del Valon ice cave represents the southernmost record for *I. ravasinii* (Fig 1B). The distributions of both species overlap in the western part of the Venetian Prealps (Schönhofer et al. 2015). Our data are in agreement with this finding (Fig. 1B). Despite being sympatric, to our knowledge this is the first known case of coexistence of these two troglophilic *Ischyropsalis* species within the same cave.

Ischyropsalis ravasinii appears to prosper inside the Buso del Valon ice cave, forming a large population and being the most abundant representative of the local harvestman fauna. Additionally, the lack of specimens collected outside the cave corroborates the strong affinity of *I. ravasinii* for subterranean habitats. However,

the population of *I. ravasinii* is not uniformly distributed inside the Buso del Valon ice cave. This species shows a marked preference for the micro-habitat formed by the thick scree in the central part of the cave, being most abundant near the border of the permanent ice during both the warm and cold seasons. Most of the specimens were sampled from the central part of the cave, including juveniles belonging to all six instars and all the adults. A similar distribution pattern seems to be followed by *I. strandi* although on a smaller scale. *Ischyropsalis ravasinii* is a troglophilic-hygrophilic species strictly bound to high humidity to deposit eggs (Juberthie 1964, 1965; and observations with specimens in captivity). It needs a cool environment of about 4–6 °C to remain active as observed with captive specimens. The interstitial spaces within the rocky debris may contribute to retain the suitable humidity and temperature for the harvestmen survival throughout the year. The scree likely represents the most stable micro-habitat inside the cave in both the seasons, consequently serving as an ideal habitat for egg deposition and juvenile development. Other areas of the cave pose colder or drier conditions, at least for a part of the year (Fig. 2A), or lack a coverage of debris that can be used as a refuge, thus being a less suitable habitat for this species.

Our data suggests a conspicuous difference in the seasonal distribution of *I. ravasinii* in the scree-covered area of the Buso del Valon ice cave. During the warm season, when the slightly warmer temperatures and the reduced extension of the ice coverage allows surface activity, *I. ravasinii* seems to be similarly present in both superficial and deep layers within the scree, with a preference for being close below the surface. In contrast, very few specimens were sampled on the surface during the cold season, most collections occurred in the DPS trap only. Lower surface temperatures and the presence of a larger and thicker layer of ice and snow in comparison to the warm season, most likely hinder the surface activity of *I. ravasinii* during the cold season. Such conditions may force *I. ravasinii* to move deeper into the scree where the micro-climatic conditions remain more suitable.

Martens (1969) reports that the reproductive season in *Ischyropsalis* spp. extends from spring to early summer, with egg hatching in late summer/autumn. Similar results were observed by us with *I. ravasinii* eggs hatched in captivity. Accordingly, the presence of the highest number of early instars (IN 1–3) collected in the cave during the cold season, together with the low numbers of adults, supports this hypothesis. The high percentage of the subadult IN 5 found during the warm season also suggests that the final maturation of this species occurs mainly during the warmer period. Therefore, it is likely that juvenile *I. ravasinii* need at least nine months to reach adulthood and probably even longer. Such hypotheses are in line with our experimental observations with specimens raised in captivity. After reaching their maturation, adults of some *Ischyropsalis* species may live for several months (e.g. *I. kollari*, see Martens 1969). The records of adults collected in the Buso del Valon ice cave during both the warm and cold periods also support this hypothesis. Dead adults of this species have occasionally been found in caves during the early summer months (July and August, Petri I. personal observation 2020) and captive adults have survived several months

before dying. Such findings imply that the life cycle of *I. ravasinii* extends for more than one year, possibly around two or more years, as reported for other *Ischyropsalis* species (see Juberthie 1968) and experimentally tested by us hatching and raising specimens in captivity.

Conclusions

The present study offers new data on the spatio-temporal distribution of the troglophilic harvestmen *I. ravasinii* adapted to living in cold subterranean environments. We investigated for the first time the ecological and seasonal preferences for microhabitats in *I. ravasinii* and we report additional data on its life cycle. Despite being the dominant harvestman in the cave, this species is absent in nearby cavities, including artificial tunnels, which are instead occupied by *I. strandi*. Since *I. ravasinii* seems to strongly rely on the presence of stable, humid and cool habitat, the Buso del Valon ice cave may provide refuge for this species similarly to other local frigophilic arthropods. In addition, the typology of substrate seems to play an important role in its survivability, the wide majority of individuals being collected in the scree-covered area of the cave.

Due to its strict bond with specific environmental conditions *I. ravasinii* may be strongly affected by even limited changes occurring to its habitat. Following the rise in temperatures related to climate change, and the consequent progressive reduction of the internal ice body, the conditions of the microhabitats inside the Buso del Valon ice cave are changing at a fast pace (Latella et al. 2019). Such changes may threat the species survivability similarly to what is occurring to other arthropods living in ice caves (Mammola et al. 2019; Howarth 2021). Additional studies on the ecology of *I. ravasinii* in the Buso del Valon ice cave may help us to explore how this or other frigophilic subterranean arthropods face the long-term effects of climate change.

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Eupera troglobia sp. nov.: the first troglobitic bivalve from the Americas (Mollusca, Bivalvia, Sphaeriidae)

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Abstract

Eupera troglobia **sp. nov.** is the first fully described troglobitic bivalve discovered in the Americas, and possibly the second in the world. The troglobitic designation is based on the lack of pigmentation, reduction in size and shell thickness, and large and few offspring. Unlike its relatives that live in the roots of the water hyacinth, this new species is attached to the walls of the caves with a byssus. The anatomical study shows some peculiarities in comparison with the congeners, especially the simplicity of the foot, the siphons and the gut. This discovery is a new contribution to the efforts for the conservation of subterranean habitats in Brazil.

Keywords

Anatomy, cavernicolous life, conservation, Mollusca, new species, Systematics

Introduction

Troglobitic animals are restricted to subterranean habitats and therefore unable to establish viable populations in surface habitats. Such organisms are adapted to living under stressful conditions, such as lack of light and scarcity of food. As for mollusks, the typical troglobitic mollusk usually exhibits the following characteristics: (1) absent or reduced pigmentation, (2) absent or reduced eyes, (3) some reduction in shell size (reduction in size compared to their epigean relatives) (Simone, in press). These characteristics have

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been idealized using the most common cave-dwelling taxon – Gastropoda. In the Americas, only gastropods have been classified as troglobites. Especially in South America, most of them are freshwater gastropods, but some terrestrial gastropods are also known (Simone and Moracchioli 1994; Simone 2012, 2018, 2019; Simone and Salvador 2021).

The only other class of mollusks that occurs outside the ocean is the bivalves (Bivalvia). The occurrence of bivalves in subterranean habitats is sparsely documented. There are few reports of external species being transported to or living in caves, such as some species from the genera *Pisidium* Pfeiffer, 1821 (Grego et al. 2019) and *Euglesa* Jenyns, 1832 (Turbanov et al. 2016) (both Sphaeriidae). Recently, an interesting bivalve species was described for Germany (*P. interstitialis* Boessneck, Groh & Richling, 2020), which has an exclusively interstitial lifestyle and thus lives outside open waters (Groh et al. 2020). However, exclusively subterranean species are rare in this group. The only undoubtedly obligate cave bivalve belongs to the dreissenid genus *Congeria* Partsch, 1835, which occurs mainly in southeastern Europe (Bilandžija et al. 2013, 2021; Chong et al. 2016). Furthermore, the small bivalves of the genera *Pisidium* and *Euglesa* could also be cave-bound species, although this "status" certainly deserves further studies to confirm or refute it. Four species of *Pisidium* have been observed only in caves: one from Turkey and three from caves in the Caucasus Mountains in Abkhazia (Bole and Velkovrh 1986). In addition, three species of *Euglesa* have also been found only in caves, two from Georgia (in the region of Imereti and Abkhazia) and one from Russia and Georgia (Turbanov et al. 2016). It is interesting to note that the rarity of bivalves in caves may be due to their greatly reduced mobility combined with their general inability to tolerate hypoxic habitats (Culver 2012). Therefore, it is unlikely that the rarity of bivalves living only in caves is due to the lack of potential surface ancestors. Surprisingly, small bivalves that can be classified as troglobites were collected from Casa de Pedra cave, Lagoa da Confusão municipality, Tocantins, central region of Brazil.

The examination of this material revealed that it belongs to the sphaerid genus *Eupera* Bourguignat, 1854. The genus is relatively widespread throughout South America and is normally found in the roots of water hyacinth [*Eichhornia crassipes* (Mart.) Solms – Pontederiaceae], an aquatic plant that floats in calm freshwater environments. A total of 11 valid species of *Eupera* occur in South America east of the Andes (Simone 2006), all of which have characteristic shell pigmentation with dark brown spots in a beige ground color (Mansur and Veitenheimer 1975).

Bivalves have eyes in only a few lineages, the Sphaeriidae are not among them, so the absence of eyes in *Eupera* cannot be considered. However, the collected specimen has the other characteristics to be classified as a troglobite: Absence of pigment, fragile shell, and small size. All of these characteristics are described in detail below.

The first records of specimens of the cave clam described here appeared in a 2006 technical report of the National Center for Cave Exploration and Conservation (Centro Nacional de Pesquisa e Conservação de Cavernas – **CECAV**) (Silva 2006). In that report, a faunal survey of Casa de Pedra Cave was presented, focusing on invertebrates (Figs 33–35). Although the author presented some photographs of the clam, little attention was paid to this species, which was thought to have been carried into the cave by the water. In 2010, one of the authors of this paper (RLF) accessed the report and

considered the possibility that this species might be a troglobite due to its depigmentation. He searched for sampled specimens but was unable to find any in the collection where the remaining specimens were deposited (ISLA/UFLA), suggesting that specimens were not collected on this occasion. In 2021, a team visited the cave specifically to search for such shells. Although the cave was partially flooded (see Habitat and Ecological Notes section), the researchers were able to find specimens that are described here.

This paper addresses the official description of the first fully described troglobitic mollusk from the Americas, which may be the second mollusk group with cave-bound representatives, apart from the dreissenid genus *Congeria* (See in Discussion the other sphaeriids mentioned above). The study includes detailed anatomical and conchological investigations as a basis for comparison with other congeneric species.

Material and methods

The material was received for taxonomical and anatomical study already fixed in 70% ethanol, with no previous narcotization method. They were mainly deposited in the MZSP collection, with paratypes selected for other indicated institutions. The dissection procedures and terminology are standard (Simone et al. 2015), with the specimens dissected under dissecting stereomicroscope, immersed in the fixative. Digital photos of the dissecting steps were obtained by connected digital camera. SEM examination was also standard, with shells covered by gold; due to the fragility of the shells, some of them were broke during this process. The anatomical drawings were obtained with the aid of a camera lucida connected to the microscope. Each drawing (as well as the description) is a synopsis of the examination of several specimens.

The following abbreviations are used in the anatomical descriptions and figures

aa anterior adductor muscle;
ae possible aesthete scar;
al anterior lateral hinge tooth;
an anus;
ap genital aperture;
ar anterior pedal retractor muscle;
as anterior hinge socket;
au auricle;
bf byssal furrow;
by byssus;
ca cardinal hinge teeth;
cc cerebral commissure;
ce cerebral ganglion;
ci ciliary gill connection;
cv cerebrovisceral connective;
dd duct to digestive diverticula;
dg digestive diverticula (gland);
em embryos inside visceral brood pouch;
es esophagus;
ex excurrent siphon;
fb foot base;
fg gill food groove;
ft foot;
hf hinge mantle fold;
id inner demibranch;
if mantle border inner fold;
in intestine;
ip inner hemipalp;
is incurrent siphon;
ki kidney;
li ligament;
mb mantle border;
mf mantle border middle fold;
mo mouth;
nv nerve;
od outer demibranch;
of mantle border outer fold;
op outer hemipalp;

pa posterior adductor muscle;
pc pericardium;
pe periostracum;
pg pedal ganglia;
pl posterior lateral hinge tooth;
pm pallial muscle;
pn pallial line;
pp palp;
pr posterior pedal retractor muscle;
ps posterior hinge socket;
rt rectum;
sh shell;
ss style sac;
st stomach;
su suspensory membrane;
sy statocyst;
um umbonal region;
un fusion between mantle lobes;
ur nephropore;
ve ventricle;
vg visceral ganglia;
yo young specimen inside marsupium of inner demibranch.

Additional abbreviations:

ISLA/UFLA Coleção de invertebrados Subterrâneos de Lavras – Universidade Federal de Lavras;
MNRJ Museu Nacional da Universidade Federal do Rio de Janeiro;
MZSP Museu de Zoologia da Universidade de São Paulo, Brazil;
USNM National Museum of Natural History, Smithsonian Institution, Washington DC, USA.

Additional abbreviatures:

LV left valve;
RV right valve;
sh shell(s);
spm specimen(s) in alcohol;
W width.

Taxonomy

Family Sphaeriidae

Genus *Eupera* Bourguignat, 1854

Type species. *Pisidium moquinianum* Bourguignat, 1854, Monotypy; = *Eupera bahiensis* (Spix in A. J. Wagner, 1827).

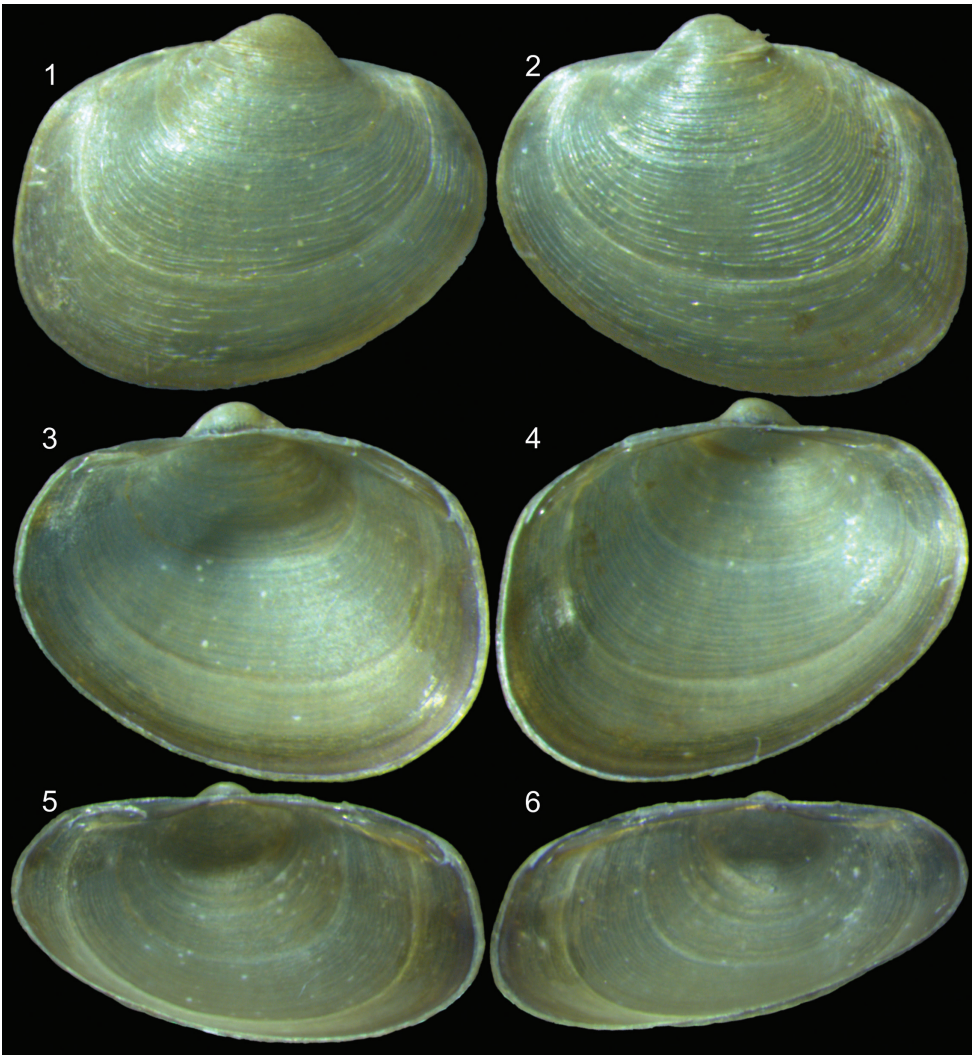
***Eupera troglobia* sp. nov.**
http://zoobank.org/5B33B18C-F5F7-47BD-AF9F-EFD9305166A7
Figs 1–30

Material examined. ***Holotype.*** MZSP 155717. ***Paratypes*** MZSP 155716, 12 specimens, MNRJ 23647, 1 specimen, USNM, 1 specimen, all from type locality.

Type locality. Brazil. Tocantins; Lagoa da Confusão, Casa da Pedra cave, 10°49'28.4"S, 49°37'16.5"W [Ferreira col., 3.viii.2021].

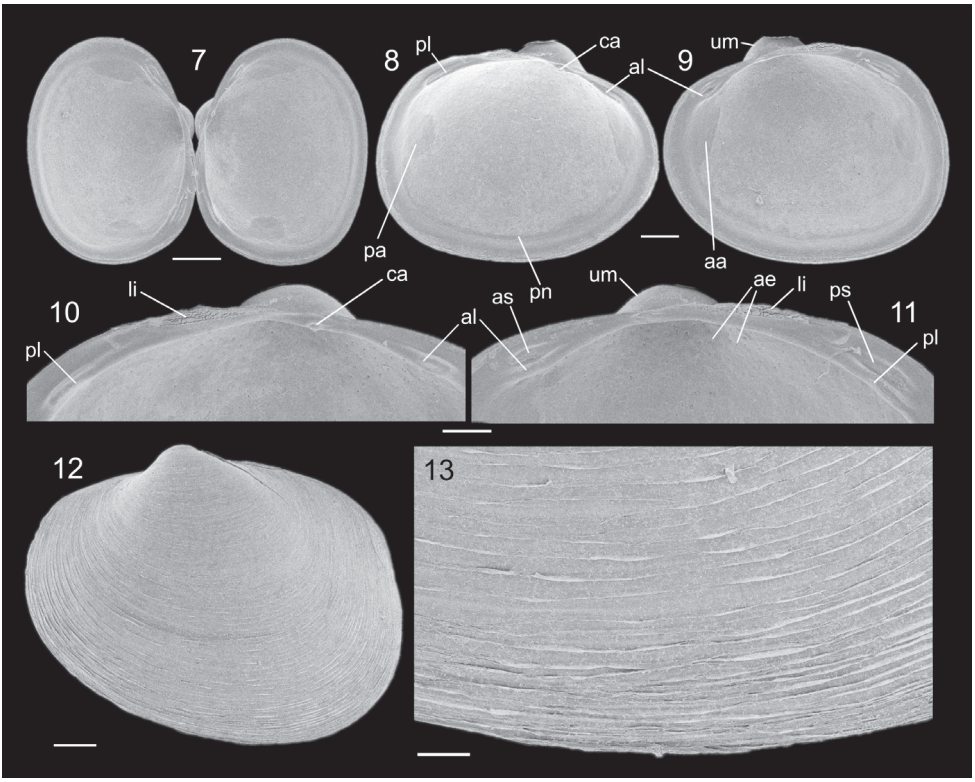
Diagnosis. Adult size ~4.5 mm. Lacking pigmentation in shell and soft parts. Shell very fragile, translucent, light yellow.

Description. ***Shell*** (Figs 1–18). Adult shell ~4.5 mm. Equivalve; height ~80% of length; width ~60% of length. Walls thin, fragile, translucent. Anterior edge rounded, smaller than posterior edge; ventral edge rounded in medium specimens (Figs 7–9) to slightly ascendent in larger specimens (Figs 1–4, 12); posterior edge almost straight in



Figures 1–6. *Eupera troglobia* holotype MZSP 155717 shell (L 4.3 mm), right valve in left column, left valve in right column **1** outer right view **2** outer left view **3** inner left view **4** inner right view **5** inner left-slightly ventral view **6** inner right-slightly ventral view.

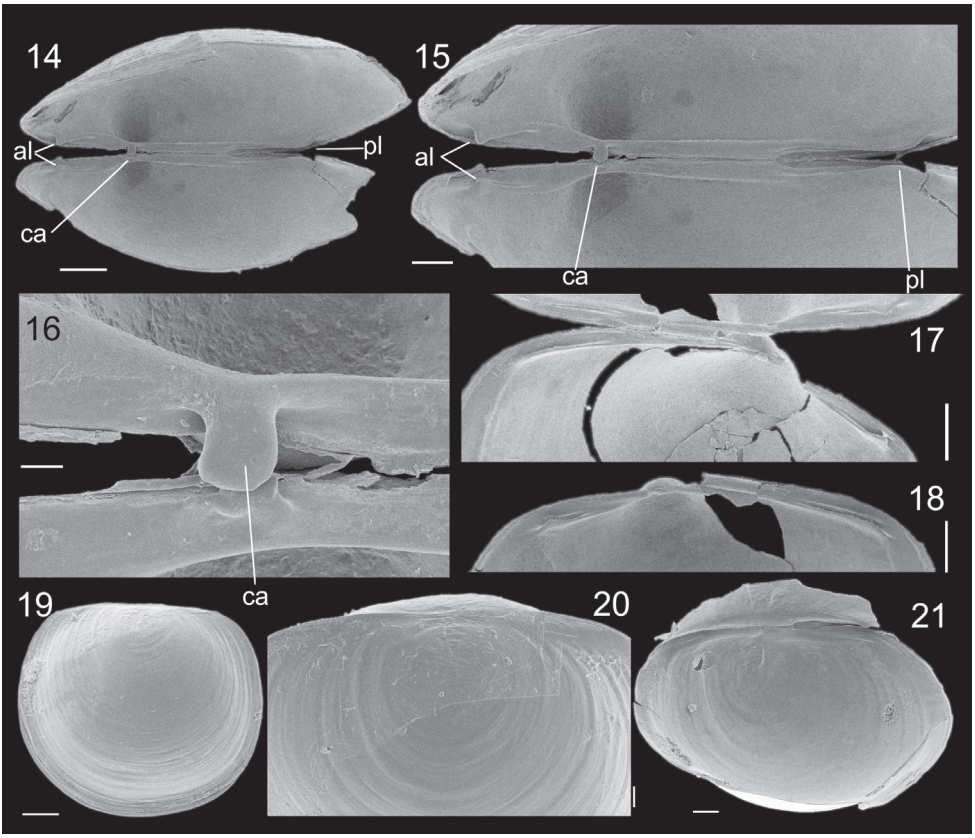
its middle level; dorsal edge weakly convex, almost straight. Color light yellow to light greenish yellow (Figs 1–6). Outer surface opaque. Sculpture of uniform concentric growth lines (Figs 1, 2, 12, 13); ~15 per mm; each line alternating in height along its length (Fig. 13), but mostly 4–5 times taller than wide; interspaces ~10 times wider than each line. Growth lines usually continuous from anterior up to posterior hinge region (Figs 1, 2, 12). Umbo (um) slightly prosogyrate, central, protruding ~10% height beyond hinge level (Figs 1–4, 8–9), occupying ~20% of dorsal edge. Hinge with small, blunt cardinal tooth (ca) in LV, ~1.5 times longer than tall, tip rounded (Figs 14–16), shallow correspondent socket in RV (Fig. 16); anterior (al) and posterior (pl) lateral teeth relatively equidistant from cardinal tooth (Figs 4–6, 7–11, 14–18), similar to each other, in both valves; located in anterior and posterior ends of hinge edge, in blunt angle preceding anterior ad posterior slopes; each lateral tooth ~4-times longer than wide, parallel to hinge edge; anterior pair of tooth usually with anterior small beak (Figs 14–15: al, 17–18); both lateral teeth of LV slightly more ventral than



Figures 7–13. *Eupera troglobia* shell SEM images of paratypes 153866 **7** specimen #7, both valves connected, opened ~120°, ventral view **8** #7, left valve, inner left view **9** #7, right valve, inner left view **10** #7, left valve, detail of hinge region, inner left view **11** #7, right valve, detail of hinge region, inner right view **12** specimen #8, left valve, outer left view **13** same, detail of surface on middle region of ventral edge. Scale bars: 500 µm (**7, 12**), 300 µm (**8**), 200 µm (**10, 13**).

lateral teeth of RV, encasing ventrally to them; both lateral teeth of RV with narrow socket lying dorsally for counterparts of LV (Figs 5, 9, 11, 18). Inner surface glossy; scar of anterior adductor muscle (aa) occupying ~5% of entire inner valve surface, ~twice taller than wide, elliptic, slightly larger than scar of posterior adductor muscle (pa) (Figs 3–4, 7–9). Pallial line continuous, simple, connecting both adductor muscle scars; relatively broad; located along ventral edge ~15% of total height distant from it. Inner surface possessing minute pits, possibly of aesthetes (Figs 10–11: ae).

Main muscle system (Figs 22, 24, 27). Anterior adductor muscle (aa) with elliptic transverse section, dorso-ventral height of slightly twice anterior-posterior width; located close to blunt angulation between dorsal and anterior shell edges. Posterior



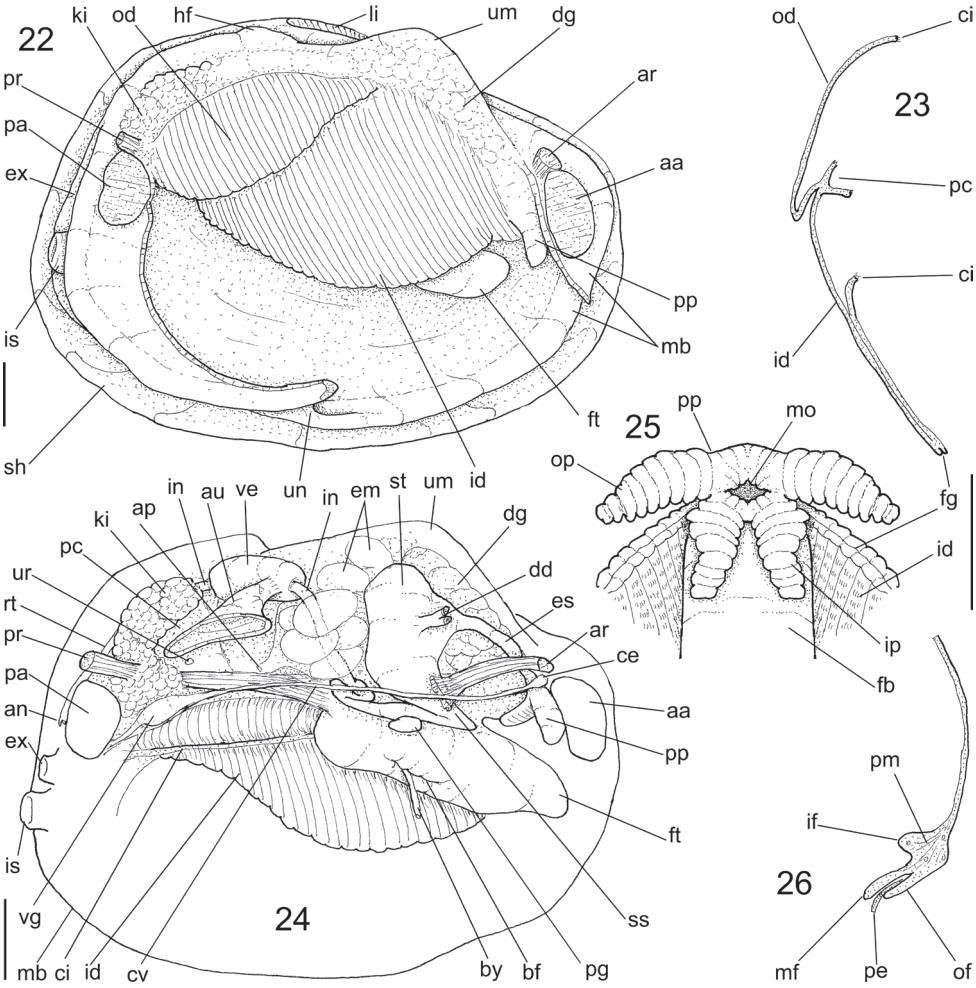
Figures 14–21. *Eupera troglobia* shell SEM images of paratypes 153866 **14** specimen #8 (part damaged), valves opened ~40°, ventral view **15** same, higher magnification **16** same, higher magnification, region of cardinal tooth **17** specimen #9 (part damaged), valves opened ~60°, ventral view, mainly showing hinge of left valve **18** same, right valve **19** shell of young specimen extracted from gill's marsupium, left valve, outer left view **20** same, detail of umbo showing prodissoconch in its center, left-slightly dorsal view **21** another intra marsupial specimen (part damaged), right valve, inner ventral-slightly left view, focus on hinge, part of left valve still attached by ligament. Scale bars: 500 μm (**14, 17, 18**), 300 μm (**15**), 100 μm (**19**), 50 μm (**16, 21**), 30 μm (**20**).

adductor muscle (pa) slightly smaller and located slightly more ventrally than anterior muscle. Pair of anterior pedal retractor muscles (ar) originated just dorsal to anterior adductor muscle in elliptic area equivalent to ~15% of that of anterior adductor muscle; running towards posterior relatively narrow, along ~20% of shell length, attached to adjacent visceral integument; insertion splaying in antero-dorsal foot base. No detectable protractor pedal muscle. Pair of posterior pedal retractor muscles (pr) originating similarly as anterior pedal muscle, but dorsally to posterior adductor muscle; running narrow anteriorly along ~50% of shell length, as central base to local visceral mass and attached to adjacent visceral integument; insertion splaying in postero-dorsal side of pedal base.

Foot (Fig. 24: ft). Cylindric, ~3-times longer than wide in contracted condition; ~half projected anteriorly beyond its base. Posterior region ventrally bulged, rounded. Anterior end bluntly tapering. Byssal furrow (bf) narrow, occupying ~1/4 of middle region of ventral foot surface. Byssus (by) as single, narrow, yellow thread.

Mantle (Figs 22, 26, 27). Mantle lobe thin, translucent, thickened only in edges. Colorless. Edges of both lobes fused with each other in region of anterior adductor muscle, and in region posterior to middle level of ventral edge; fusion provided by inner fold. Mantle edge in non-fused region (Fig. 26) with narrow, flattened outer (of) and middle (mf) folds, no papilla or special structures detectable; inner fold (if) located in inner base of middle fold, with ~half of remaining folds height, as wide as tall. Pallial musculature (pm) thin, present in base of three folds. Fusion between posterior half of mantle edges (un) simple. Incurrent siphon (Fig. 27: is) simple, cylindric, walls weakly muscular; distal edges simple, lacking papillae; length in retracted condition ~5% of shell length, ~twice longer than wide. Excurrent siphon (ex) similar to, but ~30% smaller than incurrent siphon; preserved inverted in several specimens. Siphonal musculature immersed in local mantel edges, lacking detectable bundles, neither producing pallial sinus in shell. Gill suspensory membrane (su) connected by cilia in posterior end of gill, membrane-like separating completely incurrent from excurrent chambers (Fig. 27).

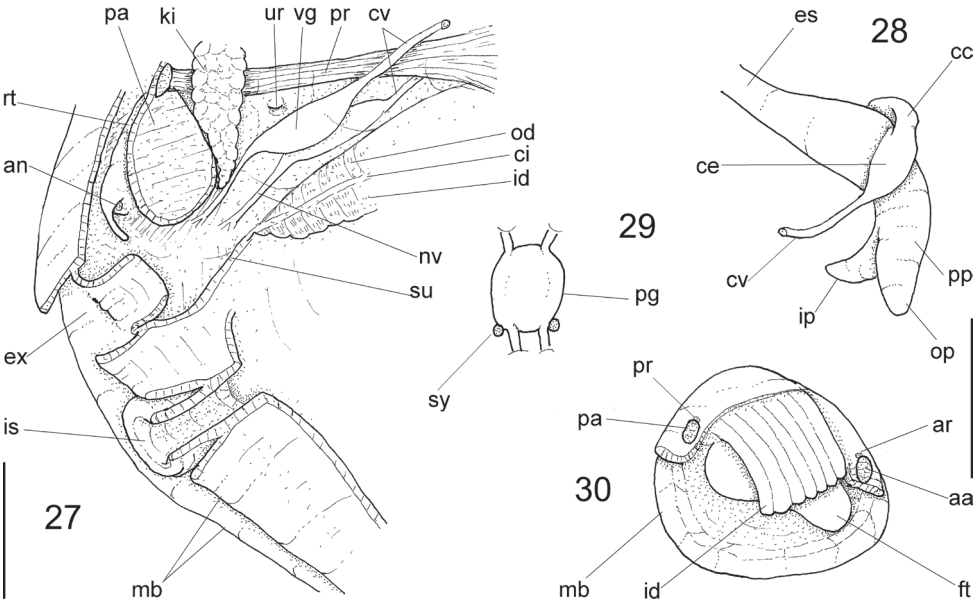
Pallial cavity (Fig. 22). Outer demibranchs (od) with ~1/4 of shell height in its middle region; tapering gradually towards anterior, up to middle level of inner demibranch dorsal edge; tapering subtly towards posterior; lamellae very narrow, with ventral curve covering small region of inner demibranch dorsal edge (Fig. 23: od); dorsal connection with visceral mass via cilia (ci). Inner demibranchs (di) wide, area ~double as outer demibranchs; anterior region slightly wider than half of shell height, gradually tapering towards posterior up to certain distance from posterior adductor muscle; transversely folded; descendent lamella (Fig. 23: id) simple, very narrow, free from ascendent lamella; ascendent lamella with ~70% of descendent lamella length; narrow food groove (fg) in inner demibranch ventral edge; inner demibranch connections with visceral mass and its counterpart (in posterior half – Fig. 24) via cilia (ci). Inner demibranch serving as marsupium of ~6–8 young specimens (Fig. 31: yo), detailed below. Pair of palps (Figs 24, 25: pp) small (~half of anterior adductor muscle area), located just posterior to anterior adductor muscle; outer hemipalps (op) ~3-times longer than



Figures 22–26. *Eupera troglobia* anatomical drawings **22** whole right view, right valve and part of right mantle lobe removed **23** gill, transverse section in its middle level **24** whole right view, right gill removed, visceral structures seen as in situ if region was transparent, peripherical structures with only topology indicated **25** palp region, ventral view, hemipalps slightly deflected **26** mantle edge, transverse section in middle level of ventral edge. Scale bars: 0.5 mm.

wide, 8–10 strong transverse folds, from edge to edge (even protruding beyond edges); tapering distally; folds ending before mouth area (mo), keeping smooth perioral area; inner hemipalp (ip) similar to outer hemipalp, but slightly smaller, usually placed close to anterior region of visceral mass.

Visceral mass (Fig. 24). All visceral structures white. Stomach (st) occupying most of anterior half, disposed anterior-ventrally. Digestive diverticular (dg), lying along anterior region of stomach. Gonad and genital structures occupying anterior ~half of posterior half of visceral mass, covering posterior surface of stomach. Reno-pericardial



Figures 27–30. *Eupera troglobia* anatomical drawings **27** peri-siphonal, posterior region, right view, right gill and mantle lobe removed, siphons longitudinally sectioned, visceral structures seen as in situ **28** transition between palps and esophagus, right view, with concern to cerebral ganglia **29** pedal ganglia, dorsal view **30** gross anatomy of young specimen from gill brood pouch, right view, right mantle lobe removed. Scale bars: 0.5 mm.

structures occupying posterior half of posterior half of visceral mass, up to posterior adductor muscle. Details below.

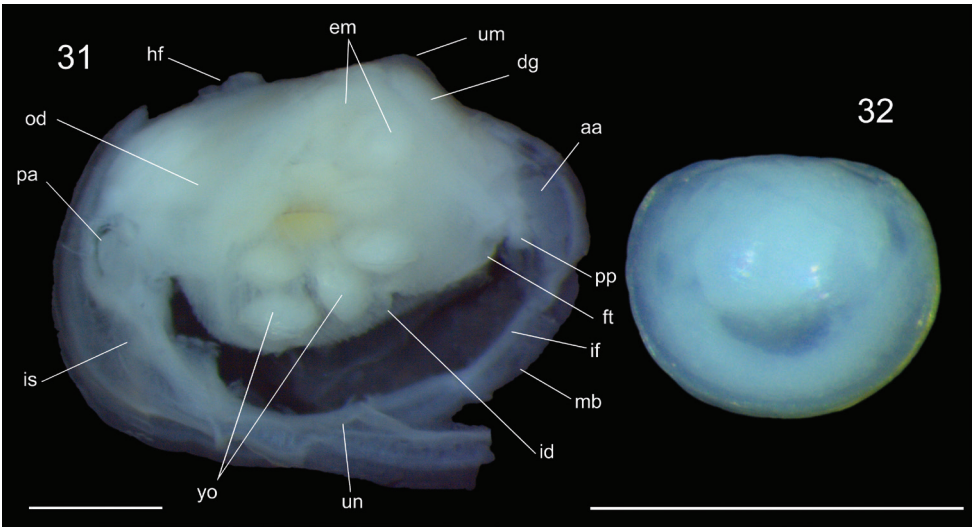
Circulatory and excretory systems (Figs 24, 27). Heart occupying anterior half of reno-pericardial area. Ventricle (ve) large, as dorsal structure, totally surrounding intestine; wall thick. Anterior and posterior aortas initially running attached to adjacent intestine. Pair of auricles (au) connected to anterior region of ventricle, each one conic, running towards ventral and lateral; connecting to central region of gills. Kidneys (ki) as posterior half of reno-pericardial volume, connected anterior and dorsal surface of posterior adductor muscle; anterior region hollow, as urinary chamber; nephropore (ur) as single, small slit located in ventro-anterior surface of supra-branchial chamber; posterior region mostly filled by white, solid renal tissue.

Digestive system (Fig. 24). Palps (pp) and mouth (mo) (Fig. 25) described above (pallial cavity). Esophagus (es) simple, narrow, running along ~20% of shell length from posterior region of anterior adductor muscle towards posterior and dorsal, inserting in anterior surface of stomach between its middle and dorsal thirds. Stomach (st) large, dorsally rounded, ventrally tapering towards ventral and anterior up to anterior region of foot base. No clear separation between intestine and style sac (ss). Duct to digestive diverticula (dd) located in center of both gastric lateral walls. Stomach inner surface simple, lacking chambers and large folds; gastric shield thin, located in postero-

dorsal region. Intestine (in) subtly running posteriorly and dorsally after style sac end, flanking dorsal surface of foot base; short zigzag only in its middle level; intestinal length slightly larger than shell length; in pericardial region crossing directly, gradually directing ventrally and posteriorly up to posterior side of posterior adductor muscle, initially immersed in pallial edge tissue; after short distance running on supra-anal chamber (Fig. 27). Anus (an) simple, sessile, located between posterior and ventral surface of posterior adductor muscle; large anal papilla in middle of anal dorsal edge (Fig. 27: an).

Reproductive system (Fig. 24). Gonad white, solid, small, mostly located in lateral regions of stomach. Large hollow brood pouch as posterior 2/3 of genital structures, full of large embryos (em); brood pouch tapering towards ventral and posterior, opening in both sides in small orifice (ap) located in middle level of suprabranchial chamber.

Central nervous system (Figs 24, 27–29). Pair of cerebral ganglia (ce) located in region dorsal to mouth, each one ~1/20 of anterior adductor muscle size. Each ganglion (Fig. 28: ce) elliptical, ~twice longer than wide; cerebral commissure (cc) wide, as long as each ganglion. Pair of pedal ganglia (Fig. 29: pg) located in middle level of pedal base; both totally fused with each other, forming spheric mass; pair of statocysts (sy) very small, located in posterior side of pedal ganglia; both pedal ganglia slightly larger than one cerebral ganglion. Pair of visceral ganglia (Fig. 27: vg) located slightly anterior to posterior adductor muscle central side; each ganglion fusiform, ~3 times longer than wide, located very close to each other, with pedal commissure very short; each visceral ganglion slightly larger than each cerebral ganglion; posteriorly single large nerve running towards posterior, ventrally to posterior adductor muscle (nv).



Figures 31–32. *Eupera troglobia* development, paratype MZSP 153866 pregnant specimen (2–3) **31** whole right view, shell and part of right mantle mole removed, young specimens (yo) seen in marsupium of inner demibranch by translucency **32** young specimen extracted from marsupium, right view. Scale bars: 1 mm.

Development. Large embryos found in gonadal brood pouch located inside visceral mass (Fig. 24: em). Embryos coming out by genital orifice (Fig. 24: ap), located in middle level of inner demibranchs. Both inner demibranchs serving as external branchial brood pouches (Fig. 31: yo), becoming full of young specimens (yo) in their internal area between both lamellae. Young specimens with prodissoconch of ~0.2 mm (Fig. 20), growing up to teleoconch becoming ~3-times larger than prodissoconch (Figs 19, 32), with ~0.7 mm. Prodissoconch almost plane, circular, smooth (Figs 19, 20); teleoconch possessing only concentric undulations and growth lines (Figs 19, 32); valves translucent (Fig. 32). Young intra-brood pouch specimen rounded, slightly flattened, dorsal region almost straight, umbos not protruding (Figs 19, 32); shell lacking teeth in hinge and with almost no inner muscular scars (Fig. 21). Gross anatomy of these young specimens (Fig. 30) with very small adductor muscles (aa, pa), with anterior slightly more ventral; mantle lobes edges (mb) not fused with each other, lacking siphons; gill with only inner demibranch visible (id), relatively squared, possessing 7–8 transverse folds only; foot lacking visible byssal furrow.

Material examined. Types.

Measurements (in mm). ***Holotype*** MZSP 155717 (Figs 1–6): 4.3 by 3.5; Paratypes MZSP 153866: #7 (Figs 7–11): 2.3 by 1.9; #8 (Fig. 12): 4.5 by 3.6.

Etymology. The specific epithet refers to the troglobitic mode of life of the animal, being an adjective in the feminine nominative singular.

Habitat. Specimens of *Eupera troglobia* sp. nov. were only observed in the Casa de Pedra cave, and are possibly endemic to this cave (Fig. 33A, B). The Casa de Pedra cave comprises a cave in limestone from the Couto Magalhães Carbonatic Formation, associated with the Neoproterozoic basement of the Baixo Araguaia Supergroup, which, in addition to the limestones, presents subordinate phyllites, slates, metargilites, metarenites and quartzites (Pereira and Morais 2012). The climate of the region is tropical, with two distinct periods: a dry season, between May and September, and a rainy season, between October and April, with a total annual rainfall around 1750 mm (Martins et al. 2002).

The cave has 1,038 meters of total length, with predominantly ellipsoidal conduits. There are few speleothems, in addition to thick allochthonous sediments on the cave floor. The cave is inserted in a limestone outcrop located close to the Lagoa da Confusão karstic lake (Figs 33C, 34A), which overflows during the rainy season, flooding part of the flood plain surrounding it. In such periods (October to April), most cave conduits are completely filled with water. On the other hand, the cave becomes dry during the dry season, as few intermittent dams are present.

A visit paid to the cave in August 2021, revealed the cave partially flooded, with most conduits inaccessible. The main entrance gallery was filled with water, which was still forming a small lake outside the cave (Fig. 34A). Reaching the deepest areas inside the cave through the main entrance was impossible in that moment, but since there is another entrance in the middle of the cave (Figs 34B, 35A), the inner portions were accessible (Fig. 35B).

Individuals of *Eupera troglobia* sp. nov. were found associated to a consolidated sediment deposit in a deeper portion of the cave (Figs 33D, 35C). Many specimens

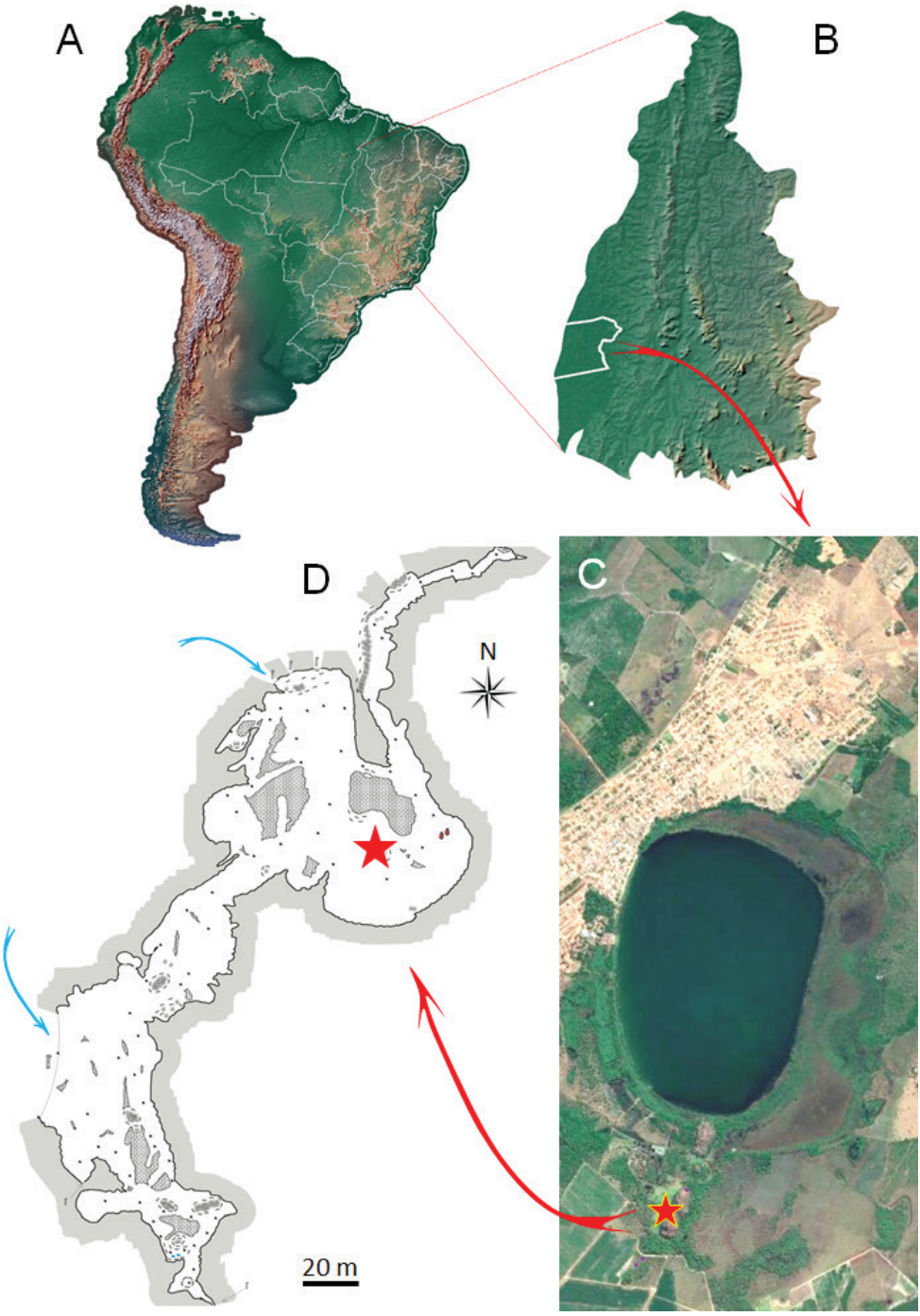


Figure 33. Location of the Casa de Pedra cave **A** South America, with Brazilian states highlighted **B** Tocantins state with Lagoa da Confusão municipality highlighted in white lines **C** aerial view from the Lagoa da Confusão region, where the urban area and the karst lake are visible; red star indicating the location of the Casa de Pedra cave **D** Casa de Pedra cave; red star indicating the location of *Eupera troglobia* sp. nov.

Figure 34. Lagoa da Confusão karst area **A** limestone outcrops close to the Lagoa da Confusão karst lake. The dark blue indicates the floodplain (not flooded in the moment of the photograph) and the dark blue indicates those flooded areas. The arrow indicates the main entrance of the Casa de Pedra cave **B** outcrop where the Casa de Pedra cave is located; 1. indicates the main entrance of the cave; 2. Upper entrance.

were adhered to the sediments already exposed to the air (Fig. 35E, F), while others were still under water (Fig. 35D, H, G). However, it is important to note that the cave was still drying up, so all specimens would be exposed to the air. Considering that the cave remains out of water during at least three months a year, the individuals do survive during all this period somehow avoiding desiccation. The only known cave-restricted clam species, all belonging to the genus *Congeria*, from caves in the Dinaric Alps, also exhibit this behavior, presenting a notable tolerance to air exposure (Jovanović et al. 2016). Interestingly, in the case of all three *Congeria* species, only part of their popula-

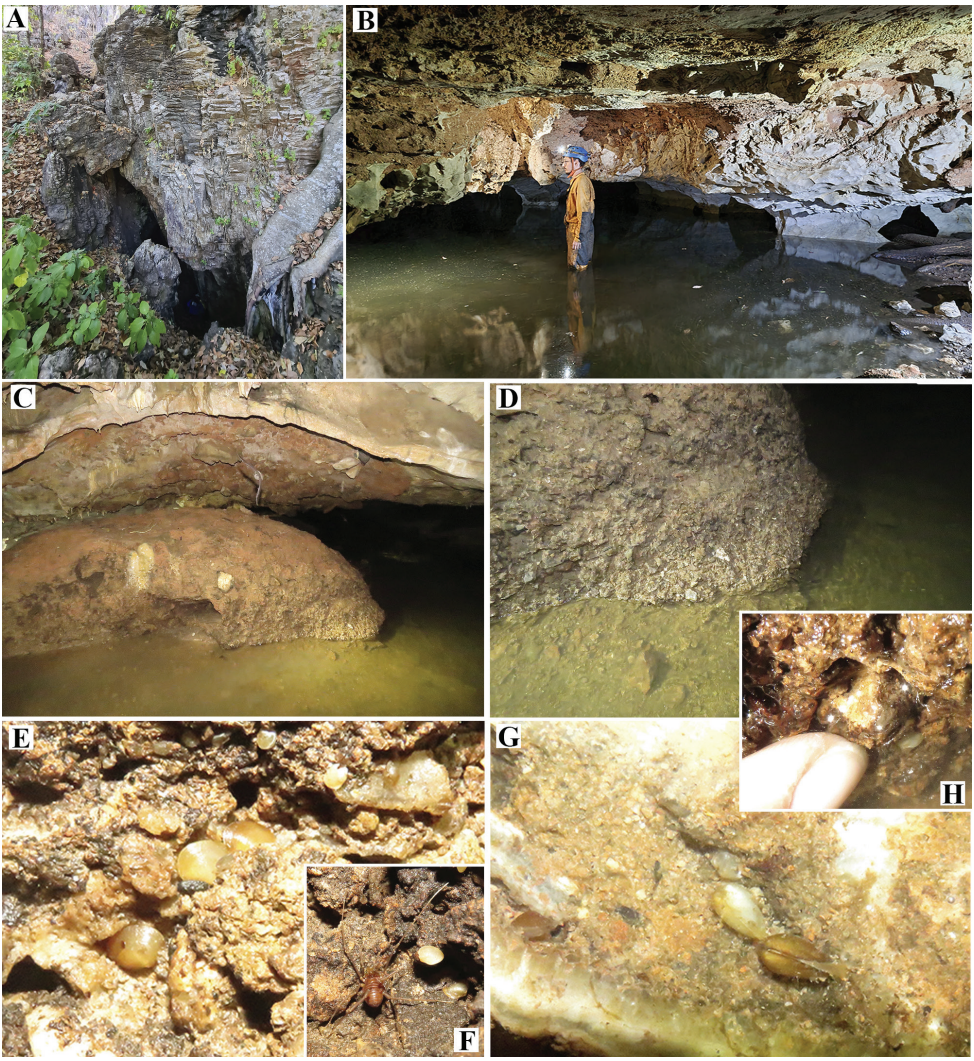


Figure 35. Casa de Pedra cave **A** secondary upper entrance of the cave **B** cave chamber that was partially flooded **C** area inside the cave where specimens of *E. troglobia* sp. nov. were found **D** detail of the consolidated sediment indicating the area where submerged specimens of *E. troglobia* sp. nov. were found **E** *E. troglobia* sp. nov. specimens in situ exposed to the air **F** same, detail of an air exposed specimen, with a harvestman (*Eusarcus* sp.) near it **G** in situ submerged specimens of *E. troglobia* sp. nov. **H** location of a submerged specimen of *E. troglobia* sp. nov.

tions becomes exposed during dry periods, and most part of the population remains underwater in such periods. Furthermore, even considering that *Congeria* specimens are able to tolerate air exposure for periods as 2 months, some individuals were observed still active, with their shells open and inhalant and exhalant syphons extruded (Jovanović et al. 2016). In the case of *E. troglobia* sp. nov. the single visit paid to the

cave does not make it possible to form any hypothesis regarding the individuals' behavior along the air exposure (e.g., whether they remain active or not). Accordingly, it is highly recommendable that further studies investigate the biology and life cycle of this species. It is worth mentioning that Silva (2006), in her report from the present cave, mentioned the presence of clams also associated to root masses pending from the cave ceiling during their survey (in the dry period). In that case, all specimens were also exposed to the air, and there were only few small ponds inside the cave, apparently devoid of clams.

During the clam sampling in August 2021, some hydrochemical parameters were evaluated, both inside the cave and in the epigean lake (Lagoa da Confusão lake), which floods to the cave during rainy periods. The parameters inside the cave were quite distinct from those from the external lake: cave waters: temperature: 23.6 °C; pH 6.17; conductivity: 0.124 mS/cm; dissolved oxygen: 0.92 mg/L; TDS (total dissolved solids): 0.08 g/L; Salinity: 0.06‰; external lake: temperature: 28.1 °C; pH 7.14; conductivity: 0.017 mS/cm; dissolved oxygen: 10.35 mg/L; TDS (total dissolved solids): 0.011 g/L; Salinity: 0.01‰. It is noticeable the differences in temperature (lower inside the cave), pH (lower inside the cave), conductivity (higher inside the cave) and dissolved oxygen (much lower inside the cave). This certainly demonstrates that the species is not only able to survive in conditions quite distinct from those observed in surface waters, but also probably tolerates high levels of variation in hydrochemical parameters along the year, considering that the cave water originates from the lake flooding.

Finally, it is also worth mentioning the number of embryos found in *E. troglobia*. Although in the literature, it is usual to find the term "embryo" referring to both the true embryos and the young, such stages are, in fact, distinct. True embryos (still in ontogenetic development) are those individuals found in the visceral marsupium. Those found in the inner demibranchs are called "young", as they are already formed and the shell shows growth lines. In *E. troglobia*, there are a maximum of 10 young in each gill (~20 in total) and another 5–6 embryos in the visceral marsupium (on each side – 10–12 in total). Hence, the species presents around 30 immatures (considering both embryos and young). In the consulted literature, only the young specimens inside gills are considered. The other already studied *Eupera* species (all epigean), presented a considerably larger reported number. As an example, *E. platensis* had between 22 and 66 young specimens in gills (Ituarte 1988); *E. cubensis* between 25 and 35 (Heard 1964) and *E. klappenbachi* had between 24 and 62 (Mansur and Veitenheimer 1975). Most cave-restricted species from several distinct groups usually have k-strategies (Howarth 1983; Bellés 1992), due to the relatively stable environments that subterranean habitats usually present. Among the reproductive adaptations related to such strategy, there are a reduced number of offspring, increased offspring body size, parental care, among others. The reduced number of embryos compared to some epigean *Eupera* species, associated to the proportional large size of the young observed in the visceral marsupium of *E. troglobia*, probably represent another adaptation to the cave environment, confirming the cave-restricted status of this species.

Discussion

Taxonomical considerations

All known South American species of *Eupera* (Simone 2006) have well-defined dark spots in the central region of the valves, contrasting with the light brown background. *E. troglobia* sp. nov. is the only species that lacks these dark spots and has a shell of uniform pale yellow color, slightly distinguishing the new species from its relatives. Besides color, outline is also useful to distinguish *E. troglobia*; it is more elongate antero-posteriorly than *E. bahiensis* (Spix in Wagner 1827); *E. iguazuensis* Ituarte, 1994; *E. klappenbachi* Mansur & Veitenheimer, 1975; *E. modioliforme* (Anton, 1837); *E. primei* Klappenbach, 1967; *E. simoni* (Jousseaume, 1889) and *E. tumida* (Clessin, 1879). On the other hand, compared to *E. doellojuradoi* Klappenbach, 1962, it is not as elongate antero-posteriorly and the anterior region is not as acuminate as that of *E. guaraniana* Ituarte, 1994. The only species that has a similar outline to *E. troglobia* is *E. platensis* Doello-Jurado, 1921, from which *E. troglobia* differs in having higher and narrower umbos, and being somewhat more laterally compressed. The hinge teeth of most species of *Eupera* are more developed than those of *E. troglobia*, especially the anterior lateral tooth is almost inconspicuous, while in the other species it is thick and prominent; the only exception is *E. doellojuradoi*, which also has narrow hinge teeth.

The anatomy of *E. troglobia* is similar to the congeneric species whose anatomy is known (Mansur and Veitenheimer 1975), especially the smallness of the palps, the arrangement of the gills and the mantle margin. The most important anatomical differences from *E. klappenbachi* are the apparently proportionally smaller size of the foot and siphons; the intestine is also much less tortuous and is located entirely posterior to the stomach, whereas part of it is anterior to the stomach in *E. klappenbachi*.

The histological arrangement of *E. platensis* was detailed by Ituarte (1977). The species is revealed to be simultaneously hermaphrodite. The production of yolky eggs and the incubation of embryos and young species was well documented. As far as possible, *E. troglobia* also has similar features. About 60% of the specimens have pregnant gills, while the remaining specimens appeared still submature. Synchronous gills brooding appears to be characteristic of the entire family (Cooley and Foighil 2000), being modified to sequential in *Sphaerium* Scopoli, 1777.

The histological arrangement of *E. platensis* was described in detail by Ituarte (1977). The species is simultaneously hermaphroditic. The production of yolky eggs and the incubation of embryos and juveniles have been well documented. As far as possible, *E. troglobia* also exhibits similar characteristics. About 60% of the specimens have pregnant gills, while the remaining specimens appeared immature. Synchronous gill brooding appears to be characteristic of the entire family (Cooley and Foighil 2000), although it was modified to sequential in *Sphaerium* Scopoli, 1777.

The anatomy of other sphaeriid genera is better known, especially those from Europe. The kidney of *Eupera* looks simpler than at least those of *Pisidium* Pfeiffer, 1821, and *Sphaerium*, which have a coiled urinary chamber (Cooley and Foighil 2000; Ituarte and Korniushin 2006; Korniushin and Glaubrecht 2006; Mansur et al. 2008). Pallial

musculature is also more uniform in *Eupera*, whereas in the other genera it is concentrated at specific locations on the valves. *Eupera* is also known for the absence of a developed brood sac in the gills, which is present in the other genera (Cooley and Foighil 2000).

It is difficult to determine whether *Pisidium* and *Euglesa* are troglobitic organisms because some populations are found in caves (Turbanov et al. 2016; Grego et al. 2019; Boessneck et al. 2020; Groh et al. 2020), as mentioned in the introduction. Both genera comprise possibly the smallest freshwater bivalves and are highly simplified. In addition, their interstitial life in the sediment is usually already associated with tiny, unpigmented, and fragile shells that overlap with troglobite assemblages. Certainly, further studies of these sphaeriids could yield additional surprises about the evolution of troglobitic bivalves.

Conservation issues

The Lagoa da Confusão karst area is spatially restricted, with only four small carbonate outcrops near the lake. The outcrop farthest from the lake (about 3 km in a straight line from the lake) has suffered from limestone quarrying, which has apparently led to the destruction of the largest cave occurring there. The other three outcrops near the lake, on the other hand, have been preserved, although the surrounding landscape has been greatly altered, primarily by agriculture. There are only three known caves in these outcrops, of which Casa de Pedra Cave is the largest. The Casa de Pedra cave is used by the locals for religious purposes. Every year in November, when the cave is dry, a mass for the deceased is celebrated in the main entrance chamber. During this time, visitors leave behind a lot of trash, such as candles, plastic bottles, and more. Fortunately, most visitors stay only in the entrance chamber and do not enter the deeper parts of the cave where individuals of *Euperia troglobia* sp. nov. occur. However, it is impossible to assess whether the species is affected by such activities. Considering that it is a filter-feeding species, any contaminants released by trash left behind by visitors could be a problem for the species. Other contaminants, such as pesticides and fertilizers used on surrounding agricultural plantations, are also a problem because during rainy seasons the lake's floodplain fills up, spreading these substances over large areas, including the cave. Brazilian laws on the national speleological heritage require that a management plan be prepared for each cave that is used by people (for recreational purposes, religious purposes, etc.). Therefore, it is imperative that a study is conducted in Casa de Pedra Cave to determine if the current religious use is altering or even threatening the cave fauna, particularly the unique population of *E. troglobia* sp. nov. Based on this investigation, a management plan can be created and implemented.

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