

Description of the world's first troglobitic Pselaphini: *Geopselaphus bullonorum* sp. nov. from southern Spain (Coleoptera, Staphylinidae, Pselaphinae)

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Abstract

A new Pselaphinae rove beetle species belonging to the genus *Geopselaphus* Jeannel, 1956, *G. bullonorum* sp. nov., from a cave in south Spain is described and diagnosed. Important morphological features of the new species are listed and photographed. The new species shows a significant troglomorphy, such as the reduction of the eyes (only three unpigmented ommatidia) and an extraordinary general elongation of the body (especially the head) and appendages (antennae, maxillary palps and legs). Being the first troglomorphic Pselaphini known worldwide.

Keywords

Cave-dwelling species, new species, Pselaphitae, troglomorphism

Introduction

Geopselaphus was established by Jeannel (1956) as a subgenus of *Pselaphus* Herbst, 1792. Subsequently, it was revised by Besuchet (1961a), who raised it to the genus level, and shortly after Besuchet (1961b) included it in a key of the Iberian Pselaphini. Subsequent contributions to the Iberian fauna were the description of new species, nomenclatural changes and new records (Besuchet 1969; Löbl 1998; Pérez and Castro 2011).

The genus *Geopselaphus* currently includes 24 known species (Schülke and Smetana 2015) and it is distributed through the mountain areas of eastern and southern Iberia, northern Morocco and Algeria as well as, the Balearic Islands (Mallorca) (Besuchet 1961a, 1969). There are very few data on its biology and habitat, but specimens are usually found sifting moss or forest litter and under stones deeply buried in the soil (Español 1935; Besuchet 1961a). They can also be found in the endogean environment, using the washing soil technique combined with Berlese funnels (unpublished observations). Recently, one species (*Geopselaphus franzi* Besuchet, 1961) has been recorded from the deep subterranean environment, although most likely it is an incidental finding (Pérez and Castro 2011; Assing 2013).

During several biospeleological explorations in the Cueva de la Pileta (Benaolán, Málaga, Spain) a new species of *Geopselaphus* was discovered, which is described here. The new species has several characters typical of those living in subterranean habitats, such as reduced eyes (only three unpigmented ommatidia, likely not functional) and a general elongation of the body (especially the head) and appendages (antennae, maxillary palps and legs).

Currently, approximately 170 species of Pselaphinae are known around the world that are associated with caves and that present different degrees of the adaptation to the deep subterranean environment (Poggi et al. 1998; Yin et al. 2015). The majority of troglobitic species are concentrated in the supertribes Goniaceritae and Batrisitae, where troglomorphy is relatively common, but this adaptive characteristic is extremely rare in the supertribe Pselaphitae, which currently has only one known troglobitic representative, a tyrine balkanic species *Decumarellus sarbui* Poggi, 1994, with a marked troglomorphy that is endemic to the Peștera de la Movile cave in Romania (Poggi et al 1998; Hlaváč et al. 2008; Hlaváč et al. 2017). It is also worth mentioning that a representative of the tribe Tmesiphorini from a cave in Nepal that has been described, *Pseudophanias spinitarsis* Yin, Coulon & Bekchiev, 2015, a non-trogomorphic species with well-developed eyes and hind wings, revealing only a modest adaptation to the underground environment (Yin et al. 2015). Our finding is therefore very surprising, because as it is the second troglobitic representative of the supertribe Pselaphitae and the first known member of the tribe Pselaphini with a marked troglomorphy at a global level.

A further surprise is that the species has been found in Cueva de la Pileta, a cave discovered in 1905 (Bullón Giménez 2005), and one of the most important locations for biospeleology in the southern Iberia (Pérez et al. 2013). The cave was visited for the first time for scientific purposes by the archaeologist H. Breuil in 1912, who collected the first samples of subterranean arthropods (Jeannel and Racovitza 1914, 1918, 1929; Pérez 2014), among them two new species: *Trechus breuili* Jeannel, 1913 (Coleoptera, Carabidae) and *Iberoniscus breuili* Vandel, 1952 (Crustacea, Isopoda). Later, although far apart in time, the visits for biospeleological purposes continued from 1934 until into the 1980s, with some of the most prominent biospeleologists of the time taking part in successive sampling, e.g., C. Bolívar, H. Coiffait, J. Mateu, and A. Vandel, (Pérez et al. 2013), despite all these visits to the cave, this new species of

Geopselaphus was not detected until 2013. Currently, the cave continues to be studied and in recent years a series of interesting publications have been produced (Carabajal et al. 2012; Pérez et al. 2013; Pérez and Aguilera 2014; Miralles and Pérez 2015; García et al. 2020).

Materials and methods

This study is based on the examination of 3 specimens of *Geopselaphus* collected in the Cueva de la Pileta in south Spain. Type material of *Geopselaphus bullonorum* is deposited in the following collections:

CCH Carles Hernando private collection, Badalona, Catalonia, Spain;
MCNB Museu de Ciències Naturals, Barcelona, Catalonia, Spain.

Dissections were made using standard techniques. Genitalia were dissected from the abdomens of specimens previously softened in boiling water for 5 minutes. Contents of the abdomen were cleared using boiling 10% KOH for 2–3 minutes to remove internal tissues, and then washed in hot water before examination. After examination, genitalia were mounted on plastic transparent cards in dimethyl hydantoin formaldehyde resin (DMHF) and pinned beneath the specimen. Specimens were studied with a Leica M125 C stereomicroscope. Habitus images were taken using a Canon EOS 50D digital camera with attached Canon MP-E 65 mm f/2.8 1–5 macro lens. Male genitalia were photographed using a Canon EOS 50D digital camera attached to a Zeiss Axiostar plus compound microscope. Serial images were stacked with Helicon Focus software.

Results

Family Staphylinidae Latreille, 1802

Subfamily Pselaphinae Latreille, 1802

Genus *Geopselaphus* Jeannel, 1956

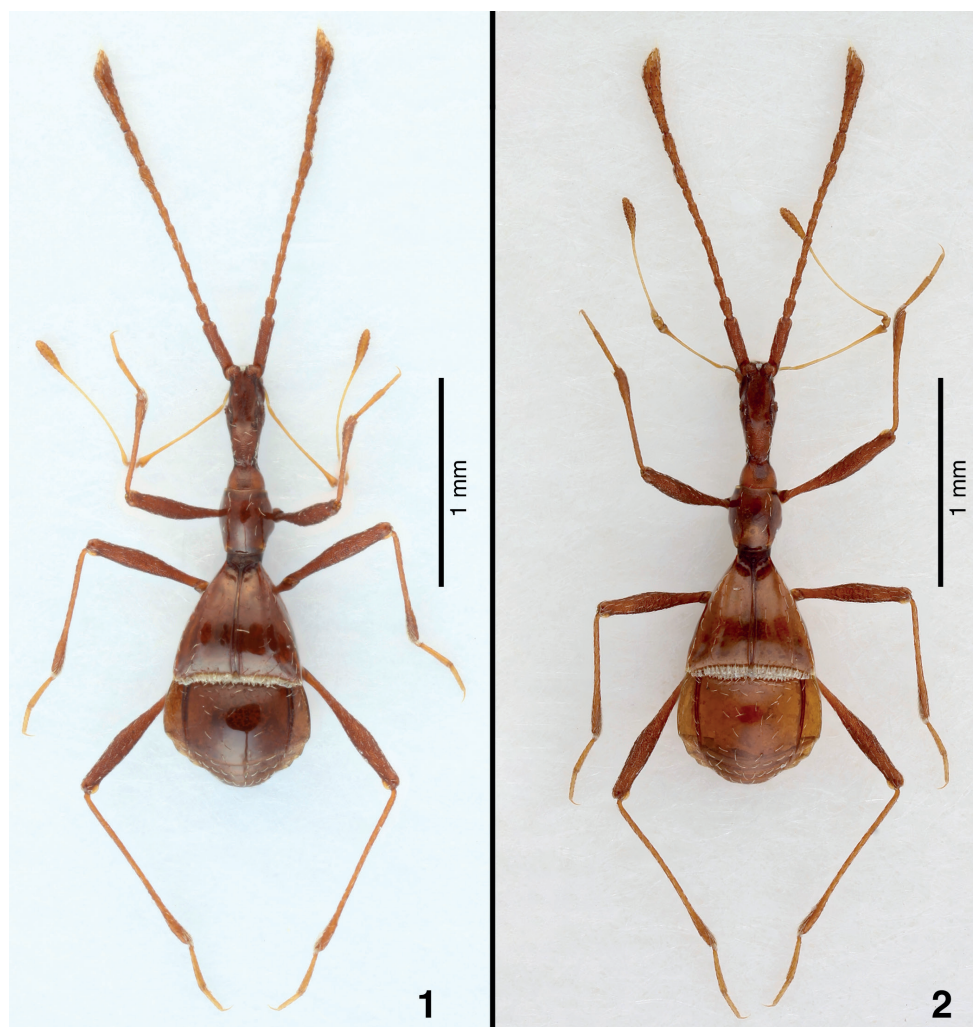
***Geopselaphus bullonorum* Hernando & Castro, sp. nov.**

<http://zoobank.org/E23CEBBD-3EED-4372-8606-D7F958A5E447>

Figs 1–10

Type locality. Cueva de la Pileta (La Pileta Cave), 36°41'28.51"N, 5°16'11.66"W, 724 m a.s.l., Sierra de Grazalema, Benaoján, Málaga, Andalusia, Spain.

Type material. *Holotype*. ♂: SPAIN: “ESP. Málaga, Benaoján / Cueva de la Pileta, 04-26-2013 / 36°41'28.51"N; 5°16'11.66"W, 724 m a.s.l. / F. Fadrique leg”, (MCNB).



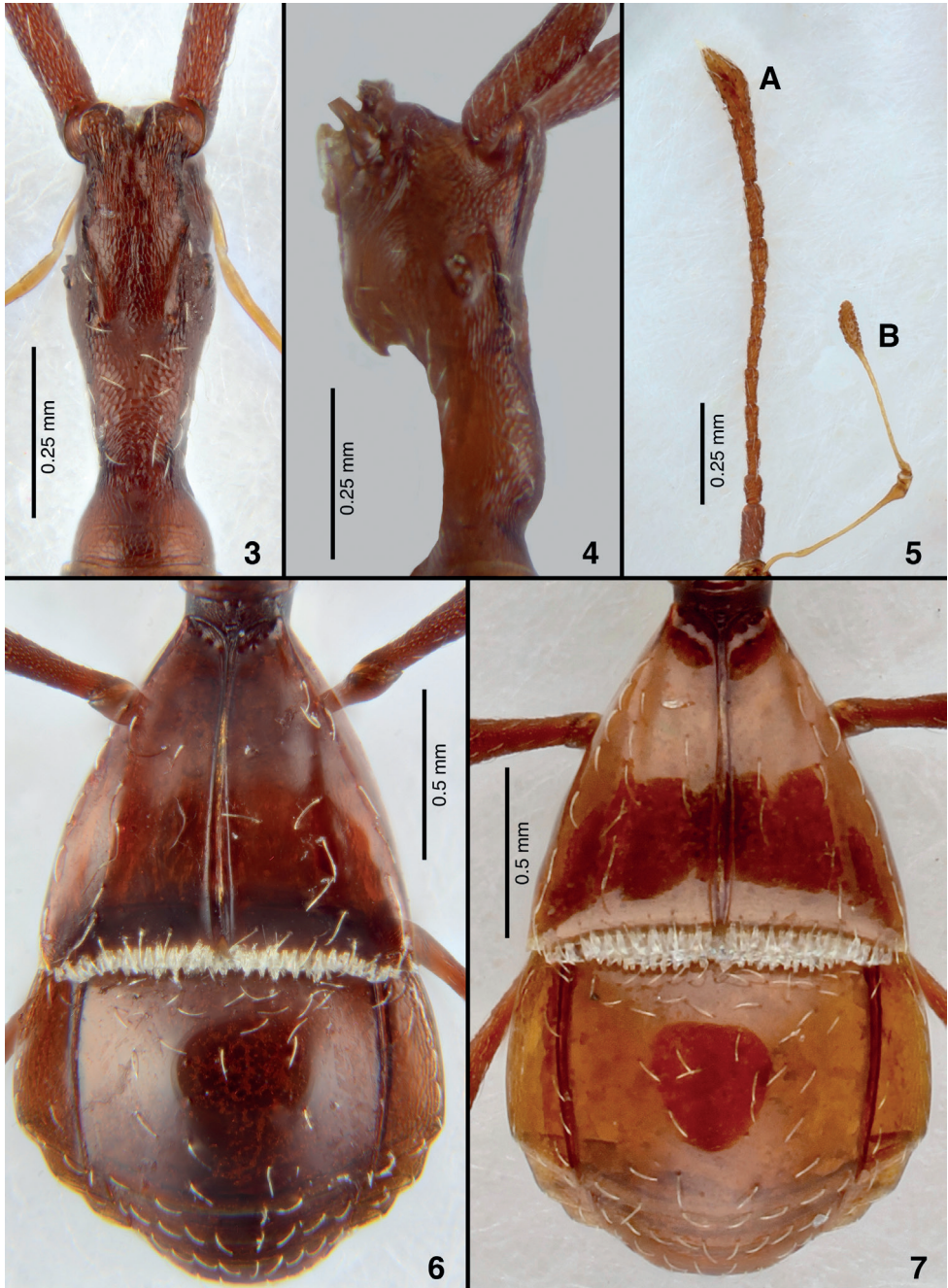
Figures 1–2. *Geopselaphus bullonorum* Hernando & Castro, sp. nov. Habitus in dorsal view **1** male **2** female.

Paratypes: 1 ♀: same data as holotype, (MCNB); 1 ♀: “ESP. Málaga, Benaoján / Cueva de la Pileta, 06-08-2015 / Sierra de Grazalema-Ronda / 36°41'28.51"N; 5°16'11.66"W, 724 m a.s.l. / T. Pérez leg.”, (CCH).

Etymology. We dedicate this new species to the Bullón family, historical owners of the Cueva de La Pileta.

Description of holotype. Habitus as in Fig. 1. Body length: 2.13 mm. Apterous and microphthalmic, with only three omatidia (probably non-functional eyes). Entirely pale brown with lighter maxillary palpi and tarsi. Body (specially the head) and appendages (antennae, maxillary palpi and legs) extraordinary elongated and slender. Pubescence of the dorsal surface consist of short suberect white setae on head, pronotum, elytra and abdomen, pubescence on the ventral surface consists of very dense clusters

of tomentose setae on the head, mesoventrite and first visible sternite, and short, suberect, white setae on the metaventricle and sternites, other suberect shorter setae on antennae and legs.



Figures 3–7. *Geopselaphus bullonorum* Hernando & Castro, sp. nov. head **3** dorsal view **4** lateral view. Head appendages **5A** antennae **5B** maxillar palpi. Elytra and abdomen in dorsal view **6** male **7** female.

Head: (Figs 3, 4) total length (0.64 mm), maximum width (0.17 mm). Extraordinarily elongate, 3.5 times longer than wide, clearly narrower than pronotum. Frons broadly grooved and distally bilobed, narrow and shallow longitudinal groove starts between both lobes, which extends back to level of eyes. Dorsolateral part with two longitudinal and parallel carinae, clearly higher in supra-ocular area and attenuated backwards, with tomentose fossae very small. Surface entirely microreticulated, dorsal pubescence composed of few short, white, semi-erect setae arranged in pair of lateral longitudinal series. In lateral view (Fig. 4), dorsal part practically flat in its anterior part, barely gibbous and slightly narrowed at posterior part. Gular protuberance projecting backward and hook-shaped (Fig. 4). Tomentose pubescence of ventral part, short and sparse, extending from gular protuberance to basal narrowing (Figs 8, 9). Eyes reduced, with only three unpigmented ommatidia, likely not functional (Fig. 3). Antennae (Fig. 5) extraordinarily long (1.57 mm) and slender, exceeding the posterior margin of elytra when bent backwards, scape cylindrical and parallel-sided, 3.2 times longer (0.28 mm) than wide (0.04 mm), pedicel cylindrical and subparallel-sided, 1.3 times longer (0.08 mm) than wide (0.04 mm), antennomeres 3–7 cylindrical, equal in length and longer (0.11 mm) than wide (0.04 mm); antennomere 8 cylindrical, with equal width, but shorter (0.06 mm) than previous five; antennomeres 9–10 cylindrical, both very similar in length and shape, wider and twice as long as previous six antennomeres; terminal antennomere very long (0.37 mm) and club-shaped, slightly asymmetrical at apex and with equal length as three previous antennomeres together. Maxillary palps extraordinarily long (1.57 mm) and slender (Fig. 5), palpomere 1 filiform, slightly curved; palpomere 2 very long (0.48 mm), filiform over practically its entire length and abruptly widened at its apical end; palpomere 3 very short and conical; palpomere 4 very long (0.73 mm), slightly arched, filiform throughout practically its entire length, progressively thickening at apex, which is club-shaped and with surface of thickened part completely covered with granules and very fine and erect pubescence.

Pronotum: Longer (0.32 mm) than wide (0.24 mm) with subparallel sides, with the anterior and posterior margins straight, the latter finely bordered, surface smooth and shiny and with two dorsal longitudinal series of setae, each consisting of 4–5 white and semi-erect setae.

Elytra: Strongly narrowed at base, practically as long (0.6 mm) as wide (0.62 mm); each elytron with three basal foveae; humeral carinae entire and well-marked, each elytron with raised, entire and well-apparent discal carinae (Figs 6, 7), with entire sutural stria; surface smooth and shiny, the pubescence on each elytron composed of three series of white and semi-erect setae, two located along the two carinae and a third very short series on the discal surface, with the posterior margins of each elytron decorated by transverse series of semi-erect setae widely spaced and on same edge with dense fringe of tomentose setae (Fig. 6).

Ventral part: Mesoventrite with two very dense clusters of tomentose setae (Fig. 8), metaventrite convex, smooth and shiny and with some widely scattered semi-erect setae (Fig. 8).

Abdomen: With tergite IV (first visible tergite) very large, clearly wider (0.71 mm) than long (0.51 mm), shorter than the elytra and clearly wider than these, with a smooth

and shiny surface and series of semi-erect setae along entire posterior margin. Sternite III (first visible sternite) with a broad transverse fringe of tomentose hairs that occupies entire basal part and with a long tuft of erect setae in central part of posterior margin (Fig. 8).

Male genitalia: Median lobe strongly acuminate at apex (Figs 10, 11) and in lateral view with the apex curved inwards (Fig. 12), internal sac with two fascicles of small spines (Fig. 11) and apical structure formed by modified spines of squamiform shape.

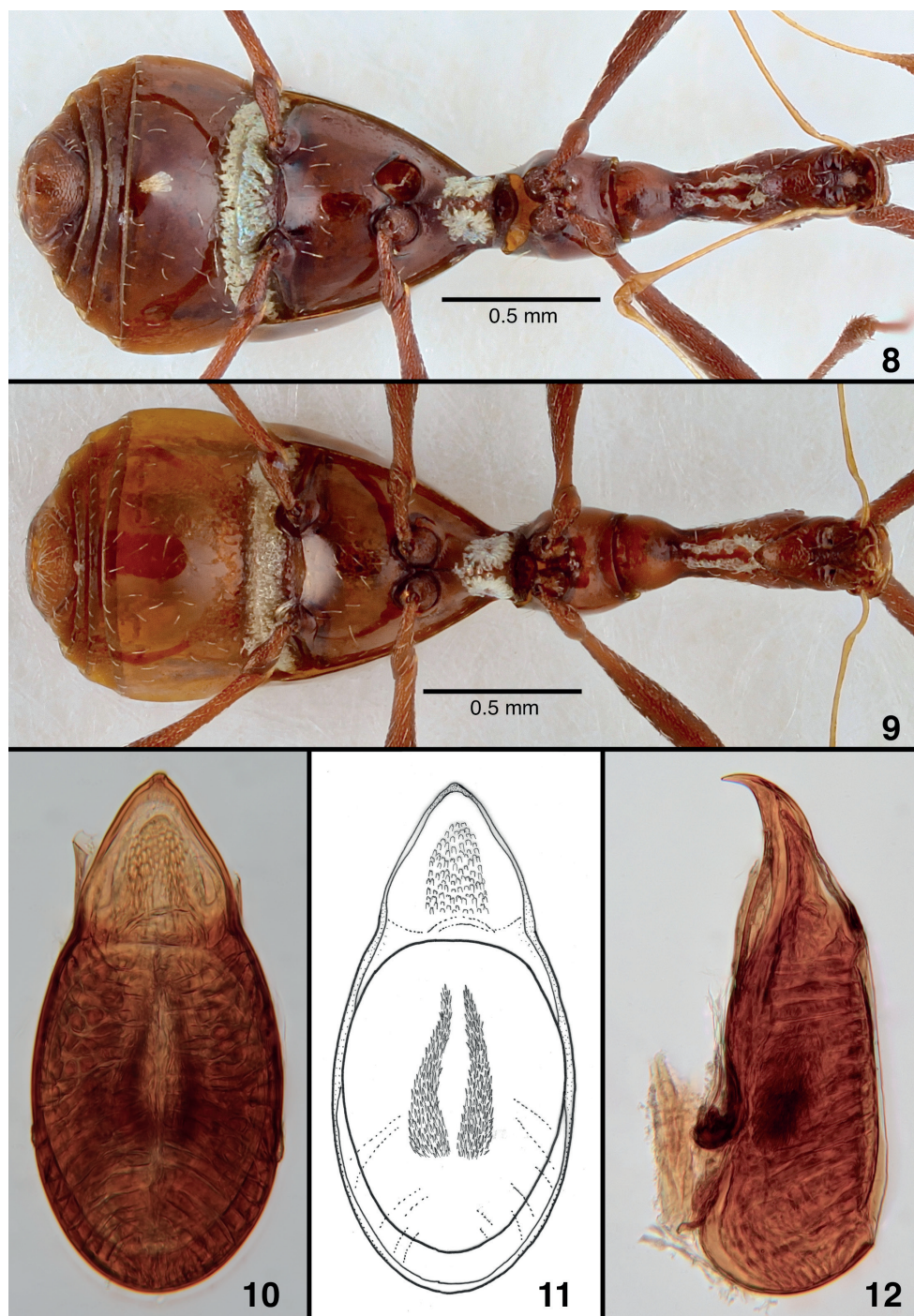
Female: General appearance similar to male (Fig. 2), but with posterior margin of the elytra with the fringe of tomentose setae broadest and more developed (Fig. 7), tergite IV clearly longer (0.55 mm), practically as long as length of elytra (Fig. 7), sternite III longer, without tuft of erect setae and rugulose at the base (Fig. 9).

Comparative notes. The new species is well distinguished from the rest of the known species of the genus by its marked troglomorphism, such as the reduction of the eyes (only three unpigmented ommatidia) and an extraordinary general elongation of the body (especially of the head) and appendages (antennae, maxillary palps and legs). It is also characterised by the small size of the tomentose foveae on the head, which are large in the rest of the known species, the elytra have a complete dorsal carina (Figs 6, 7), which is absent or slightly elevated in the rest of the known species of the genus, and the aedeagus is quite different (Figs 10–12). It has also been compared with its geographical neighbour *Geopselaphus longulus* Besuchet, 1961, an endogean species showing some elongation of the head and palps, but always in comparison with the other endogean species and without any comparison with the new species.

Based on the aedeagus, *Geopselaphus bullonorum* seems to be close to *Geopselaphus sencieri* (Coquerel, 1858), distributed in Algeria, Morocco and the extreme south of the Iberian Peninsula (Jeannel 1956), both species have an acuminate median lobe, and the internal sac is very similar, consisting of two fascicles of spines (see Besuchet 1961).

Geographic distribution. So far, only known from the typical locality.

Habitat. The Cueva de la Pileta has a total length of 2.406 m and a maximum slope of 102 m. All the specimens were captured wandering on walls in the sector called “La Gran Sima”, which is in one of the deepest parts of the cave, approximately 400 m from the cave entrance. This cave harbours a rich community of terrestrial subterranean arthropods, 32 species have been recorded so far (Pérez et al. 2013; Pérez and Aguilera 2014; Miralles and Pérez 2015), of which 12 are exclusively troglobitic. Six of them have a relatively wide distribution: *Iberoniscus breuili* Vandel, 1952, *Trichoniscus gordonii* Vandel, 1955 (Isopoda); *Lithobius motasi* Matic, 1967 (Lithobiomorphae); *Tegenaria herculea* Fage, 1931, *Troglohyphantes furcifer* (Simon, 1884) (Araneae) and *Laemostemus lederi* (Schaufuss, 1865), *Trechus breuili* Jeannel, 1913, *Speonemadus bolivari* (Jeannel, 1922) (Coleoptera); two species have a very restricted distribution and are only known from the Cueva de la Pileta and from some other caves in the same geographic area: *Cryptops longicornis* Ribaut, 1915 (Scolopendromorpha) and *Dysdera bicornis* Fage, 1931 (Araneae) and finally, with the new species describe here, four endemic species are known from Cueva de la Pileta: *Baeticoniscus bulloni* García, Miralles-Núñez & Pérez, 2020 (Isopoda); *Glomeris (Stenopleuromeris) albida* Mauriès & Vicente, 1977 (Glomerida); *Occidenchthonius bullonorum* (Caravajal, García Carrillo & Rodríguez Fernández, 2012) (Pseudoscorpiones) and *Geopselaphus bullonorum* sp. nov.



Figures 8–12. *Geopselaphus bullonorum* Hernando & Castro, sp. nov. Ventral part **8** male **9** female. Aedeagus **10–11** dorsal vision **12** lateral vision.

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Rediscovery and phylogenetic analysis of the Shelta Cave Crayfish (*Orconectes sheltae* Cooper & Cooper, 1997), a decapod (Decapoda, Cambaridae) endemic to Shelta Cave in northern Alabama, USA

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Abstract

The Shelta Cave Crayfish (*Orconectes sheltae*) is a small, cave-obligate member of the genus *Orconectes* (family Cambaridae) endemic to a single cave system—Shelta Cave—in northwest Huntsville, Madison Co., Alabama, USA. Although never abundant, this stygobiont was regularly observed in the 1960s and early 1970s before the population and aquatic community in general at Shelta Cave collapsed likely in response to groundwater contamination and the loss of energetic inputs from a Grey Bat (*Myotis grisescens*) maternity colony that abandoned the cave after installation of a poorly designed cave gate. We conducted 20 visual surveys of aquatic habitats at Shelta Cave between October 2018 and July 2021. Although the aquatic community has not recovered, we did confirm the continued existence of *O. sheltae*, which had not been observed in 31 years, with observations of an adult female on 31 May 2019 and an adult male on 28 August 2020. We conducted the first phylogenetic analyses of *O. sheltae* and discovered that the species is most closely related to other geographically proximate stygobiotic crayfishes in the genus *Cambarus* in northern Alabama than members of the genus *Orconectes*. We advocate for recognition of this species as *Cambarus sheltae* to more accurately reflect evolutionary relationships of this single-cave endemic and offer recommendations for its management, conservation, and future research, as this species remains at high risk of extinction.

Keywords

Cambarus, conservation, Crustacea, Decapoda, endangered, Malacostraca, phylogeny, population decline, short-range endemism

Introduction

Shelta Cave (Alabama Cave Survey no. AMD4) located in northwest Huntsville, Madison County, Alabama, USA was once considered one of the most biologically diverse cave systems not only in the United States but globally (Culver and Sket 2000). Twenty-four cave obligate taxa have been documented historically at Shelta Cave, including 12 troglobionts and 12 stygobionts (Cooper 1975; Hobbs and Bagley 1989; Culver and Sket 2000). The decapod fauna was exceptionally diverse with three stygobiotic crayfishes – Southern Cave Crayfish (*Orconectes australis* (Rhoades, 1941)), Alabama Cave Crayfish (*Cambarus jonesi* Hobbs & Barr, 1960), and Shelta Cave Crayfish (*O. sheltae* Cooper & Cooper, 1997) – and one shrimp – Alabama Cave Shrimp (*Palaemonias alabamiae* Smalley, 1961). However, the aquatic fauna experienced precipitous declines in the early 1970s (Cooper 1975), which has been attributed to gating of the entrances leading to the extirpation of a Grey Bat (*Myotis grisescens* Howell, 1909) summer maternity colony that provided an important energy input for the aquatic ecosystem and groundwater pollution associated with increased urbanization (Hobbs and Bagley 1989; Wilson and Robison 1993; McGregor et al. 1997; Culver 1999; Elliott 2000, 2012). Several species, including the Tennessee Cave Salamander (*Gyrinophilus pallescens* McCrady, 1954), *P. alabamiae*, and *O. sheltae*, have not been observed for several decades and have been presumed extirpated from Shelta Cave (Elliott 2005; Cooper and Cooper 2011; USFWS 2016).

Orconectes sheltae is a small, stygobiotic crayfish endemic to Shelta Cave. This rare crayfish was discovered in August 1963 after examining specimens collected for experimental studies but was not formally described until 1997 (Cooper and Cooper 1997; holotype, allotype, and morphotype are accessioned in the North Carolina State Museum of Natural Sciences with additional paratypes accessioned in the National Museum of Natural History). *Orconectes sheltae* co-occurs with *C. jonesi* and *O. australis*, but can be readily distinguished from these two crayfishes, which were historically much more abundant than *O. sheltae* (Cooper 1975; Cooper and Cooper 1997), by examination of chelae, gonopods (in form I males), and body size. *Orconectes sheltae* has narrow, elongate chelae with a long palm and that lacks conspicuous setae, the gonopod terminates in two elements – one with a twist corneous central projection and the other with a noncorneous mesial process, and body size is smallest of three species ranging 13.5–19.7 mm total carapace length of 19.7 mm (Cooper 1975; Cooper and Cooper 1997; Buhay and Crandall 2009). In *C. jonesi*, the chelae are larger, robust, and covered with conspicuous setae, the gonopod terminates in two strongly recurved elements with a corneous central project and a tapering noncorneous mesial process, and body size is intermediate between *O. sheltae* and *O. australis* ranging 15.0–23.9 mm total carapace length at Shelta Cave (Cooper 1975; Buhay and Crandall 2009). In *O. australis*, the chelae are larger but not as robust as in *C. jonesi* and also not conspicuously covered in setae, the gonopod terminates in two acute elements – a corneous central projection that is flattened basally and a noncorneous mesial process, and body size is largest of three species ranging 21.0–47.2 mm total carapace length at Shelta Cave (Cooper 1975; Buhay and Crandall 2009).

Orconectes sheltae was uncommon even before the decline in the aquatic fauna at Shelta Cave. Only 18 individuals (15 specimens collected and 3 observed) were observed over nine trips conducted between December 1963 and July 1968 (Cooper and Cooper 1997). An additional 97 individuals were observed during a long-term mark-recapture study between November 1968 and July 1975 (Cooper 1975). The most recent confirmed sighting occurred on 8 December 1988 when a form I male was captured and released in the East Hall section of the cave (Hobbs and Bagley 1989). Despite several biosurveys of the aquatic fauna conducted over the past 30+ years, no additional observations are known (Rheams et al. 1992; McGregor et al. 1994; Miller 2013). *Orconectes sheltae* is State Protected in Alabama under 220–2-.98 (Invertebrate Species Regulation) and a Priority 1 Species (Highest Conservation Concern) under the State Wildlife Action Plan (Alabama Department of Conservation and Natural Resources 2015). This species has been assessed as Critically Endangered B2ab(iii) on the IUCN Red List (Schuster et al. 2010) and Critically Imperiled (G1) by NatureServe (NatureServe 2021). However, the lack of any confirmed observations at Shelta Cave or discovery of new populations over the last 30+ years despite additional surveys has led to concerns that the species may be extinct (Buhay and Crandall 2005; Elliott 2005; Niemiller and Taylor 2019).

Here we report on the rediscovery of *O. sheltae* at Shelta Cave for the first time since 1988. We also summarize available data on cave crayfish counts at Shelta Cave over a nearly 60-year period since 1963. We also provide the first phylogenetic analysis of *O. sheltae* and propose placement of the species in the genus *Cambarus* (subgenus *Aviticambarus*) with several other cave-obligate *Cambarus* species that occur in northern Alabama.

Methods

Study area

The two vertical entrances to Shelta Cave are located within a large sinkhole in a residential area in northwest Huntsville, Alabama within the Highland Rim physiographic province and Tennessee River Watershed in northern Alabama. The 762-m cave system trends in an east-west direction under Cave Avenue, Pulaksi Pike, and several residential homes. Shelta Cave was purchased in 1967 by the National Speleological Society (NSS) to protect and preserve the diverse cave community for scientific research and conservation (Hobbs and Bagley 1989; Culver 1999). The cave is still owned and maintained as a preserve by the NSS. Shelta Cave is developed within the Mississippian-aged Tusculumbia Limestone and Fort Payne Chert undifferentiated (Rheams et al. 1992) and is characterized by three large rooms that are interconnected by short passageways (Fig. 1A). For detailed descriptions of Shelta Cave see Johnston (1933), Torode (1973), Cooper (1975), Hobbs and Bagley (1989), and Rheams et al. (1992). The largest room is Jones Hall measuring ~201 m east-west × 250 m north-south that consists of two main levels: an upper level with a substantial accumulation of breakdown slabs on the

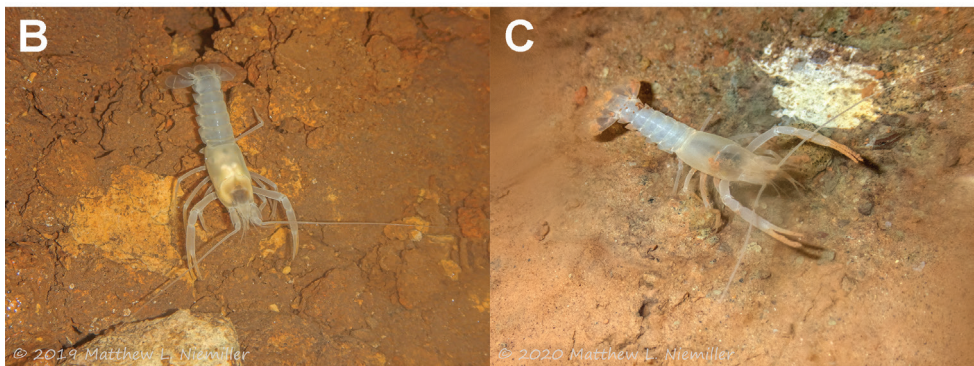
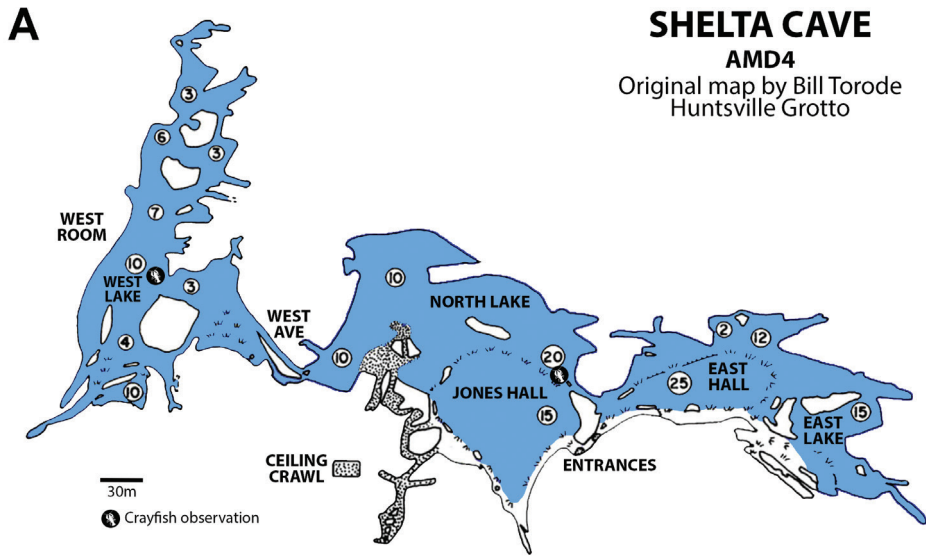


Figure 1. A Map of Shelta Cave showing the distribution of aquatic habitat during high water levels and the location of Shelta Cave Crayfish observations (black crayfish symbol) during the study including **B** a female in 2019 from North Lake and **C** a male in 2020 from West Lake. Map modified with permission of the Alabama Cave Survey.

floor and a lower level ~9 m below the upper level with a mud-cover floor and some breakdown slabs. The western area of the lower level is known as Johnson Hall. Historically, a Grey Bat summer maternity colony roosted in the northwest section of Jones Hall in an area called Bat Lake. East Hall (also called East Room) is the smallest of the three main chambers measuring ~244 m × 61 m. Miller Hall (also called West Room) is the western most chamber and is accessed from Jones Hall through Cooper's Crawl. This chamber measures ~135 m × 300 m with ceilings rarely exceeding 4.6 m and the floor characterized by large blocks of breakdown covered by thick mud. The 6.1-m pit entrances open into a high passage that connects Jones Hall with East Hall. During periods of low water levels in late summer and fall, isolated pools can be found in Jones

Hall and East Hall that gradually diminish in extent until no water is present (Cooper 1975). Miller Hall contains the only permanent water in the cave year-round – West Lake which typically maintains 0.9–1.2 m of water and up to 0.6 m of mud and silt substrate during the dry season. Grey Bats also were known to roost in this section of the cave. During winter and spring, the local water table rises, and the lower levels of the cave completely flood with groundwater becoming inaccessible. Water levels may fluctuate 10 m or more between the wet and dry seasons. Dye tracing has demonstrated that water flows from West Lake in Miller Hall to Jones Hall likely to East Hall and ultimately to Brahan Spring 3.5 km to the southeast of the cave.

Aquatic surveys

We conducted visual encounter surveys of accessible aquatic habitats (isolated pools and phreatic lakes) on 20 occasions between October 2018 and July 2021. Surveys were conducted by 1–5 researchers for 0.75–6.5 person-hours depending on water levels. During low water levels, surveys were conducted on foot in the lower levels of Jones Hall, Miller Hall, and East Hall. During high water levels, surveys were typically limited to the upper levels of Jones Hall and East Hall. We searched for aquatic life with headlamps and handheld dive lights. During high water, we also conducted snorkel surveys in Jones Hall. We made a concerted effort to capture with handheld dipnets and examine all cave crayfish observed. Select crayfish were photographed before measuring total length, carapace length, chelae length and width, and examined reproductive condition. Species identification was based on examination of chelae, gonopods, and body size (as noted above in the Introduction) and aided by photographs of type material in the North Carolina State Museum of Natural Sciences by Guenter Schuster. We also removed and retained a walking leg as a tissue sample preserved in 100% ethanol for genetic analyses. Captured crayfish were released at their point of capture after processing.

Population trends

To investigate whether cave crayfish abundance (i.e., direct visual counts) has changed over time at Shelta Cave, we compiled count data from literature sources spanning 1968–2012, including Cooper (1975), Lee (1987), Hobbs and Bagley (1989), Rheams et al. (1992), McGregor et al. (1994, 1997), Cooper and Cooper (1997), and Miller (2013). These data were combined with visual counts from our recent surveys. We employed generalized linear models (GLMs) with the census counts as the response variable and survey date as the explanatory variable for two time periods: 1) 1968–1975 during Cooper's (1975) regular crayfish surveys for his dissertation work, and 2) 1985–2021. We examined two datasets: a dataset representing visual counts for *O. sheltae* and the second which includes visual counts of all cave crayfishes observed irrespective of species. Because count data often exhibit a Poisson or negative binomial distribution and also can be zero-inflated (Linden and Mantyniemi 2011), we explored the best fit of several different distributions, including zero-inflated and non-zero-in-

lated Poisson, negative binomial, and negative binomial with NB2 parameterization [variance = $\mu(1 + \mu/k)$], using the *glmmTMB* (Brooks et al. 2017) package in R. We developed zero-inflated models using a single zero-inflation parameter but also developed hurdle models that first modeled the binary likelihood that a 0 value is observed and modeled the non-zero observations using a truncated Poisson or negative binomial model. We determined the best fitting models using AICc using the *bblme* package in R (Bolker and R Core Development Team 2017). The best fitting model was used to estimate the overall trend during each time period. We also tested for differences in abundance of each dataset between the two time periods using a Mann-Whitney test after testing for deviations from normality with a Shapiro-Wilk normality test.

DNA extraction, PCR, and sequencing

We extracted DNA from walking legs using the Qiagen DNEasy Blood and Tissue Kit according to the manufacturer’s protocol except for a few modifications. Walking legs were manually crushed using a small pestle after addition of Buffer ATL. This was followed by the addition of 40 μ L of proteinase K. The sample was incubated overnight at 56 °C, with occasional vortexing while incubating to ensure adequate mixing. After the addition of Buffer AL, the sample was incubated at 70 °C for 10 minutes. Finally, the DNA was eluted using 125 μ L of Buffer AE which had been preheated to 70 °C.

Polymerase chain reaction (PCR) was used to amplify two mitochondrial loci, 454 bp of 16S rRNA (*16S*) and 642 bp of cytochrome oxidase subunit I (*COI*). Each 25 μ L PCR reaction consisted of 12.5 μ L of GoTaq Colorless MasterMix (Promega), 1.0 μ L each of 10 μ M forward and reverse primers (Table 1), 7.5 μ L of molecular grade water, and 3.0 μ L of DNA template. Gel electrophoresis was used to confirm successful PCR amplification using an Axygen gel documentation system. PCR products were cleaned using ExoSAP-IT (Affymetrix) and sequenced in both directions using BigDye chemistry at Eurofins MWG Operon (Louisville, Kentucky) using PCR primers.

Table 1. PCR primers for amplification of two mitochondrial (*16S* and *COI*) loci in the current study.

Primer	Gene	Sequence (5'-3')	Reference
16Sar	<i>16S</i>	CGCCTGTTTATCAAAAACAT	Palumbi (1996)
16Sbr	<i>16S</i>	CCGGTCTGAACCTCAGATCACGT	Palumbi (1996)
LCO1490	<i>COI</i>	GGTCAACAAATCATAAAGATATTG	Folmer et al. (1994)
HCO2198	<i>COI</i>	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)

Phylogenetic analyses

Forward and reverse sequences were trimmed at the ends based on quality and assembled into contigs in ChromasPro v2.1.8 (Technelysium Pty Ltd, South Brisbane, Australia). Contigs were aligned using MUSCLE (Edgar 2004) in MEGA X version 10.0.5 (Kumar et al. 2018). We checked for the presence of premature stop codons

and indels in *COI* sequences, which would indicate pseudogene sequences, by converting to amino acid sequences in MEGA X. Novel sequences generated in this study were accessioned into GenBank (accession nos. ON380874–ON380893 for *COI* and ON383885–ON383904 for *16S*). We also included in our *16S* and *COI* datasets sequences for other cave and surface cambarid crayfishes available on GenBank (Suppl. material 1: Table S1) to infer phylogenetic placement of *O. sheltae* within Cambaridae and with respect to other cave crayfishes, in particular. This approach has been used in other decapods (e.g., Varela and Bracken-Grissom 2021; Varela et al. 2021). The crayfishes *Astacus astacus* (Linnaeus, 1758) and *Pacifastacus leniusculus* (Dana, 1852) both in the family Astacidae were included as outgroup taxa. We constructed gene genealogies using maximum likelihood (ML) analysis using RAxML-HPC v.9.2.10 (Stamatakis 2014). Optimal models of nucleotide substitution for each locus, including first, second, and third codon positions for *COI* and *16S*, were determined in PartitionFinder2 (Lanfear et al. 2017) using corrected Akaike's Information Criterion (AICc). ML analyses were conducted under the GTRGAMMA model and rapid bootstrapping algorithm with 10,000 bootstraps. Partitions determined by PartitionFinder2 were included in the analyses. Trees were visualized in FigTree v1.4 (Rambaut 2014). We calculated mean uncorrected pairwise distances between *O. sheltae* and other cave crayfishes occurring in northern Alabama in MEGA X (Kumar et al. 2018).

Results

Aquatic surveys

We observed 20 cave crayfish (mean \pm 1 SD: 1.3 ± 1.6 crayfish) during 12 of 20 surveys between October 2018 and July 2021. Eighteen crayfish were identified as *O. australis*. However, two individuals were identified as *O. sheltae* (Fig. 1). During a snorkel survey of North Lake in Jones Hall on 31 May 2019, MLN captured a small crayfish in ~3.7 m of water. The female crayfish measured 36 mm total length and 18 mm carapace length (Fig. 1B). The crayfish lacked first pleopods, possessed narrow and elongate chelae, and lacked prominent spines on the mesial margin of the carpus. All characters, in addition to smaller adult size, are consistent with *O. sheltae*. Developing ova also were observed internally. A second individual was captured in West Lake in Miller Hall on 28 August 2020 by MLN and NS. This form I male measured 30.5 mm total length and 14.4 mm carapace length, possessed narrow, elongate chela, and lacked prominent spines on the mesial margin of the carpus (Fig. 1C). In addition, gonopod shape was consistent with the original description of *O. sheltae* (fig. 1B, C, E, and F in Cooper and Cooper 1997).

Population trends

We assembled cave crayfish count data for 122 surveys spanning from November 1968 through July 2021 including the current study (Suppl. material 2: Table S2). Total cave

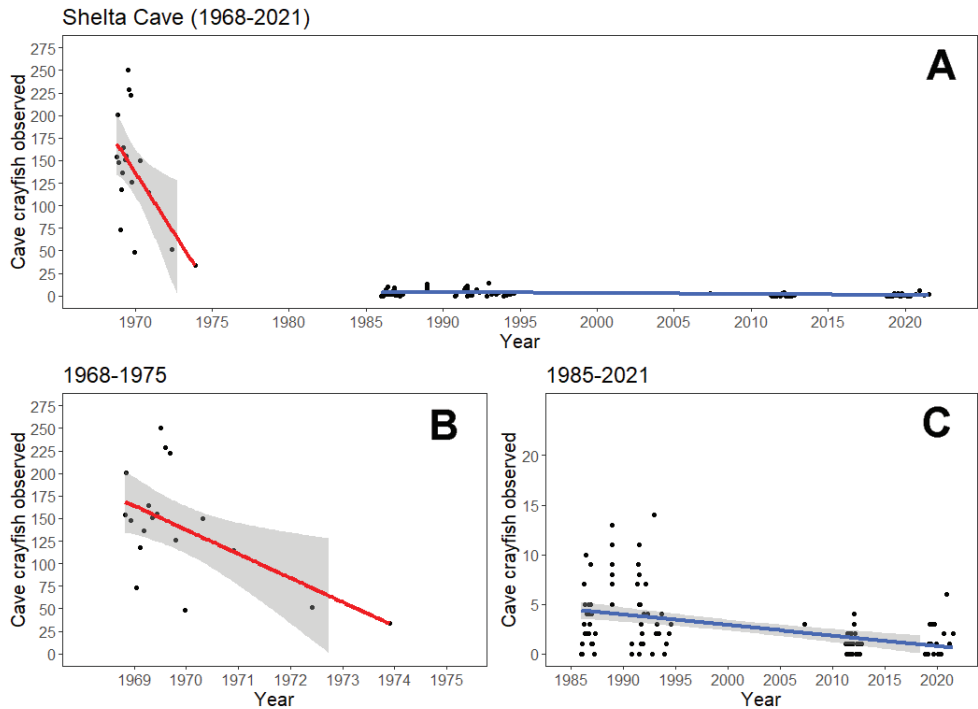


Figure 2. **A** Trends in visual census counts of all cave crayfishes (*Orconectes australis*, *Cambarus jonesi*, and *C. sheltae*) at Shelta Cave from 1968–2021, with **B** trends during the study of Cooper (1975) and **C** since 1985 also visualized. Note that the scale on the x- and y-axes differ for **B** and **C**.

crayfish abundance averaged 140.4 ± 61.9 crayfish (maximum: 250; minimum: 34) during Cooper's (1975) study over 18 surveys spanning November 1968–November 1973. *Orconectes sheltae* abundance averaged 6.2 ± 6.2 individuals (maximum: 18; minimum: 0) over the same period. Both total cave crayfish ($U = 1818$, $P < 0.001$; Fig. 2) and *O. sheltae* abundance ($U = 1907$, $P < 0.001$; Fig. 3) were drastically lower over 101 surveys spanning December 1985–July 2021 compared to the November 1968–July 1975 period. Total cave crayfish abundance averaged 2.8 ± 3.2 crayfish (maximum: 14; minimum: 0) and *O. sheltae* abundance averaged 0.03 ± 0.17 crayfish (maximum: 1; minimum: 0) over this latter period. Best fitting models (negative binomial and negative binomial with ND2 parameterization; Table 2) showed significant declines in abundance between 1968 and 1975 for all cave crayfishes (Fig. 2B) and *O. sheltae* (Fig. 3B).

Phylogenetic analyses

The *16S* and *COI* ML analyses placed *O. sheltae* in a clade with other troglomorphic *Cambarus* endemic to the Interior Low Plateau karst region in northern Alabama with strong support (Figs 4, 5). This clade includes *C. hamulatus*, *C. jonesi*, *C. laconensis*, *C. pecki*, *C. speleocoopi*, and *O. sheltae*. Mean uncorrected pairwise distances between

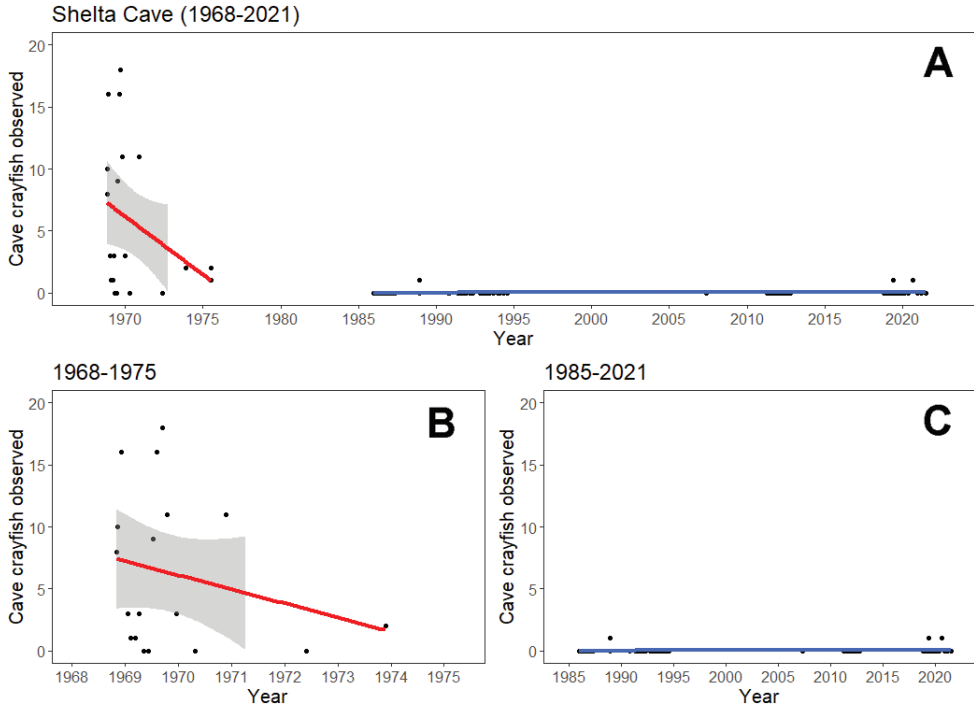


Figure 3. **A** Trends in visual census counts of *Cambarus sheltae* at Shelta Cave from 1968–2021, with **B** trends during the study of Cooper (1975) and **C** since 1985 also visualized. Note that the scale on the x-axis differs for **B** and **C**.

Table 2. Summary of parameter estimates and AICc for best model distributions (i.e., $\Delta\text{AICc} < 2$) comparing abundance (visual census counts) over time (days) at Shelta Cave for the 1968–1975 and 1985–2021 periods for all cave crayfish and for just *Cambarus sheltae*. Zero-inflation (zi.m) model parameters are included. Significance: *** - $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

Dataset	Model	AICc	Parameters
All cave crayfish			
1968–1975	Negative binomial with NB2	197.1	Intercept: 5.47***; days: -8.39e-04***; k: 7.9; df: 3
	Negative binomial	198.3	Intercept: 5.37***; days: -6.72e-04***; k: 18.2; df: 3
1985–2021	Negative binomial with NB2 parameterization	418.3	Intercept: 2.36***; days: -1.25e-04***; k: 1.7; df: 3
	Zero-inflated hurdle negative binomial with NB2	420.1	Intercept: 2.43***; days: -1.26e-04***; k: 2.1; zi.m. intercept: -3.04; df: 4
Shelta Cave Crayfish			
1968–1975	Negative binomial with NB2	120.1	Intercept: 2.30***; days: -7.45e-04; k: 1.0; df: 3
1985–2021	Gaussian	-66.6	Intercept: -0.018; days: 3.91e-06; df: 3

O. sheltae and other species in this clade ranged 5.1–7.8% and 3.0–6.2% for *COI* and *16S*, respectively. The other cave-obligate *Orconectes* form a monophyletic group more closely related to other members of *Cambarus* (Figs 4, 5). Mean uncorrected pairwise distances between *O. sheltae* and cave *Orconectes* ranged 10.6–11.9% and 6.8–7.8% for *COI* and *16S*, respectively.

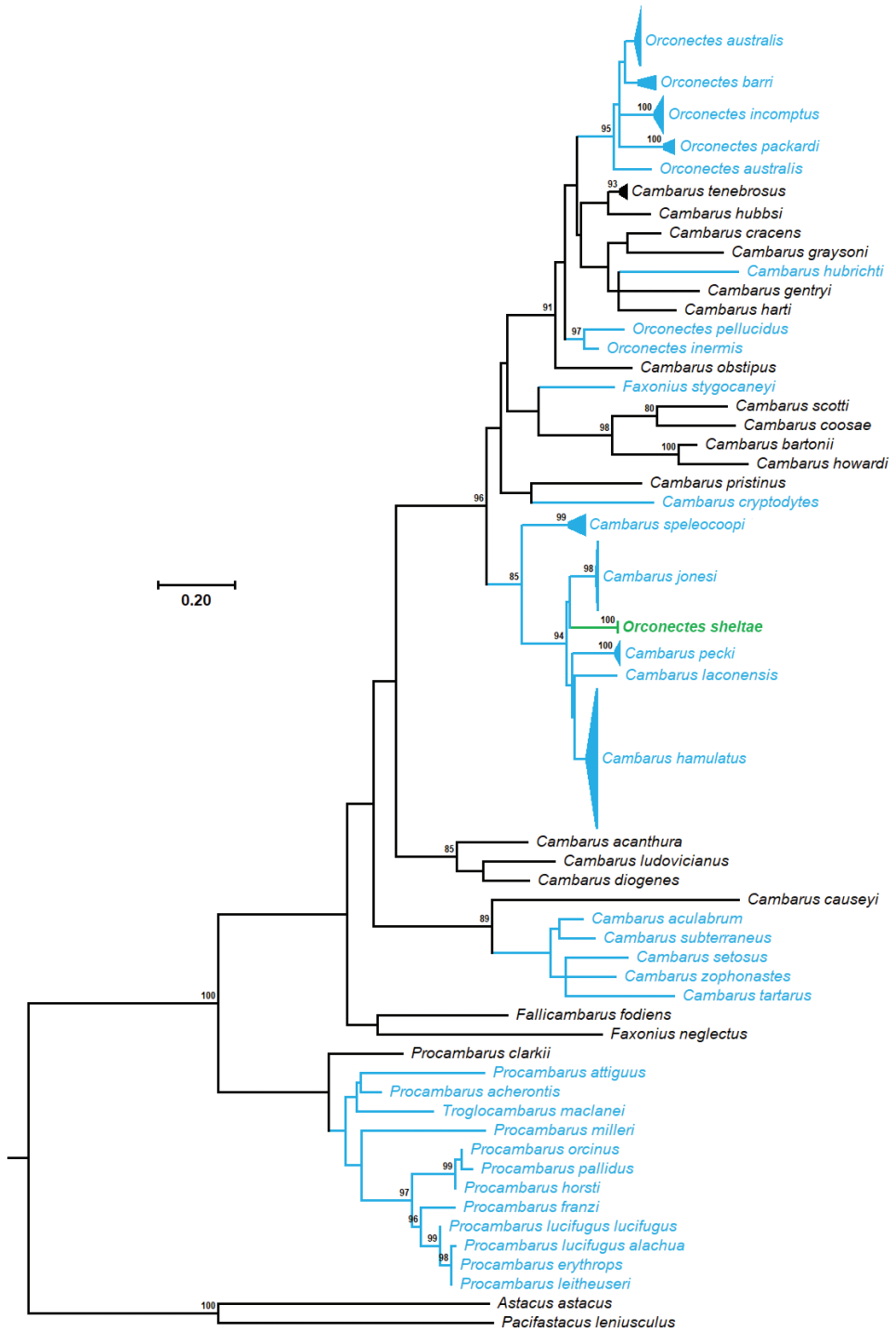


Figure 4. Maximum-likelihood phylogram showing relationships among *Orconectes sheltae* (in green) and other cave (in blue) and surface (in black) cambarid crayfishes inferred from the mitochondrial *COI* locus. Bootstrap support >80% is shown to the left of the corresponding node.

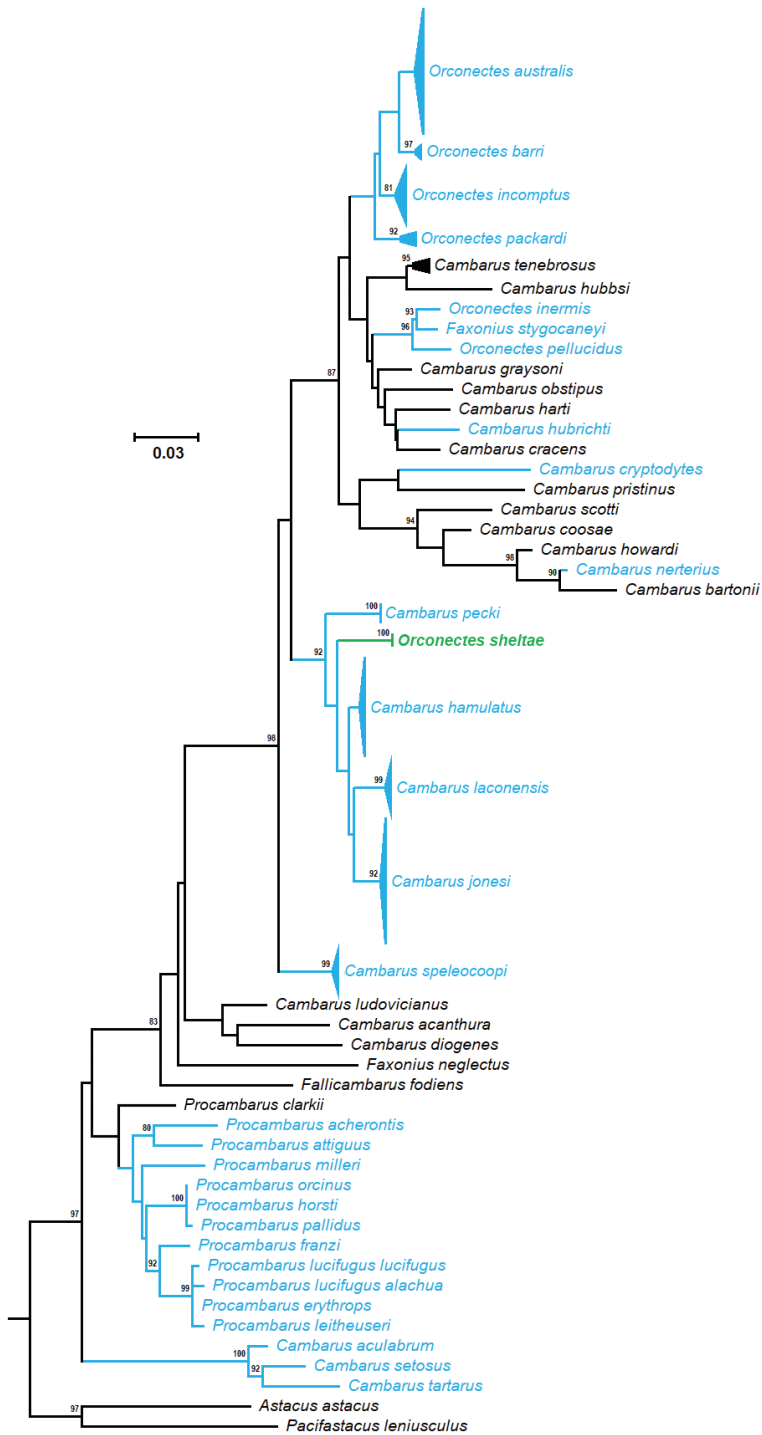


Figure 5. Maximum-likelihood phylogram showing relationships among *Orconectes sheltae* (in green) and other cave (in blue) and surface (in black) cambarid crayfishes inferred from the mitochondrial 16S ribosomal RNA locus. Bootstrap support >80% is shown to the left of the corresponding node.

Discussion

Rediscovery of *Orconectes sheltae*

The discovery of two individuals of *O. sheltae* during recent surveys in 2019–2020 demonstrates that the species is not yet extinct, as has been hypothesized by past authors (Buhay and Crandall 2005; Elliott 2005; Niemiller and Taylor 2019). However, the species remains critically imperiled and on the brink of extinction, as the population appears to be extremely small in size given only three individuals have been documented since the 1970s and overall cave crayfish abundance since 1985 at Shelta Cave is just ~2% of counts during Cooper's (1975) dissertation work in the late 1960s into the early 1970s. The rediscovery of *O. sheltae* also offers hope that other imperiled stygobiotic species thought to be extirpated may be rediscovered at Shelta Cave in the future. The federally endangered *Palaemonias alabamiae* was last observed on 24 November 1973 at Shelta Cave (Cooper 1975; Cooper and Cooper 2011) but has been documented at two new sites in the last 20 years (USFWS 2016; Niemiller et al. 2019). The salamander *Gynophylus pallencus* was never abundant at Shelta Cave or other caves in and around the metropolitan Huntsville area (Cooper 1968, 1975; Cooper and Cooper 1968, 2011; Niemiller and Niemiller 2020). This top aquatic predator has not been observed since 1968 at Shelta Cave (Cooper 1975). *Orconectes sheltae* co-occurs with two other stygobiotic crayfishes at Shelta Cave. While recent surveys have confirmed the continued persistence of *O. australis*, *Cambarus jonesi* has not been documented since 1988 (Hobbs and Bagley 1989).

Population declines in *O. sheltae* and other stygobionts at Shelta Cave have been linked to impaired water quality and reduction in energy input into the aquatic ecosystem (Hobbs and Bagley 1989; Moser and Rheams 1992; Wilson and Robison 1993; McGregor et al. 1997; Elliott 2000, 2012). Elevated levels of cadmium and the pesticides heptachlor and dieldrin have been detected in the groundwater of Shelta Cave, the latter likely associated with increased urbanization (Hobbs and Bagley 1989; Moser and Rheams 1992; McGregor et al. 1997). The pesticides likely entered via rapid infiltration through epikarst from residential areas located within the recharge basin of the cave system. The aquatic ecosystem likely also was negatively impacted by the loss of a *Myotis grisescens* maternity colony (Elliott 2000, 2012), which was an important source of organic input through deposition of guano and dead individuals. After purchasing the cave in 1967, the National Speleological Society constructed a bat-unfriendly gate in 1968. The colony was estimated at 54,000 bats in 1969, and bats were occasionally observed during the early 1970s (Cooper 1975; Cooper and Cooper 2011); however, the colony had completely disappeared by the late 1970s (Hobbs and Bagley 1989). The gate was replaced with a more appropriate design in 1981 (Hobbs and Bagley 1989) and completely removed and replaced with a high fence around the entrance in the early 2000s (Elliott 2012), but the bat colony has yet to return, and the aquatic ecosystem has yet to recover at Shelta Cave.

Phylogenetic placement and taxonomic implications

The taxonomy of crayfishes in the family Cambaridae has been based historically on morphology; however, phylogenetic relationships and evolutionary histories may be obfuscated by convergent evolution (Crandall and Fitzpatrick 1996; Taylor and Knouft 2006; Breinholt et al. 2012), which may be of particular concern when elucidating phylogenetic relationships in cave-obligate crayfishes (Sinclair et al. 2004; Buhay and Crandall 2009) and cave organisms in general (Christiansen 1961; Culver et al. 1995; Wiens et al. 2003). The taxonomy of Cambaridae continues to be in a state of flux. Several studies have uncovered lack of support for monophyly of several genera (Taylor and Knouft 2006; Johnson et al. 2011; Breinholt et al. 2012; Crandall and De Grave 2017; Stern et al. 2017; Glon et al. 2018), including *Orconectes* (Crandall and Fitzpatrick 1996; Fetzner 1996; Sinclair et al. 2004; Buhay and Crandall 2005, 2008; Owen et al. 2015; Stern et al. 2017). Recently, Crandall and De Grave (2017) presented a phylogeny based on a subset of molecular data generated in Stern et al. (2017) which showed that cave *Orconectes* form a distinct clade more closely related to members of *Cambarus*, while surface *Orconectes* are more closely related to *Barbicambarus*, *Creaserinus*, and other members of *Cambarus*. Consequently, Crandall and De Grave (2017) restricted *Orconectes* to the cave taxa, as the type species of the genus is *Orconectes inermis*, and resurrected the genus *Faxonius* Ortmann, 1905 for the surface-dwelling group. Phylogenetic relationships of cave-obligate cambarid crayfishes based on *16S* and *COI* datasets estimated in this study are similar to relationships estimated based on a maximum-likelihood analysis of partial mitochondrial (*12S*, *16S*, and *COI*) and nuclear (*28S*) sequence data by Carroll et al. (2021) and a six-locus dataset (*12S*, *16S*, *COI*, *18S*, *28S*, and histone *H3*) by Stern et al. (2017).

Our phylogenetic analyses revealed that the genus *Orconectes* as currently recognized is in need of additional taxonomic refinement, as we did not find support for inclusion of *O. sheltae* within *Orconectes*. Cooper and Cooper (1997) placed the Shelta Cave Crayfish in the genus *Orconectes* based primarily on gonopod morphology, and this classification has been followed since its description (e.g., Crandall and de Grave 2017). However, Cooper and Cooper (1997) noted that *O. sheltae* is “quite different from the other troglobitic members of the genus.” Specifically, *O. sheltae* i) lacks the first pleopods in females; ii) possesses a broad median trough of the annulus; iii) possesses elongate, narrow chelae, with a long palm and subvertical orientation; iv) has longer terminal elements of the form I male gonopod, with a greater degree of curvature and cephalocaudal flattening of the central projection; v) possesses a great depth of the cephalocaudal axis of the shaft of the gonopod proximal to the base of the central projection; vi) lacks prominent spines on the mesial margin of the carpus; and vii) is small in size, with a maximum carapace length of 19.7 mm. Our molecular results demonstrate that *O. sheltae* is a member of a clade that contains other geographically proximate cave-obligate species in northern Alabama in the genus *Cambarus*. Therefore, we advocate for recognition of this species as *Cambarus sheltae* to more accurately reflect evolutionary relationships in this taxon.

Discordance between gonopodal morphology and genetics in cave crayfishes of northern Alabama is not without precedence. Buhay and Crandall (2009) discovered

that *Procambarus pecki* was in fact also a member of *Cambarus* based on molecular analyses and recognized the species as *Cambarus pecki*. Like *O. sheltae*, *P. pecki* was noted previously to be “disjunct with other members of the genus” and once belonged to its own monotypic subgenus *Remoticambarus* (Hobbs 1972). It is becoming increasingly clear that relying solely on morphological characters is inadequate to infer species’ boundaries and taxonomic relationships in cave-obligate crayfishes because of convergent evolution on troglomorphic characters, such as reduction in eye structures, loss of pigmentation, and attenuation of antennae and limb, as well as confounding gonopodal structures. Rather, genetic data paired with geographic information, other ecological data, and diagnosable characters (outlined in Buhay and Crandall 2009) appear to be sufficient for species identification in these stygobiotic crayfishes. It is important to note that past authors have cautioned against relying solely on mitochondrial loci for inferring phylogenetic relationships, as this has the potential to yield inaccurate hypotheses in arthropods (Fontaine et al. 2007; Song et al. 2008; Leite 2012), including crayfishes (Song et al. 2008; Buhay 2009; Schubart 2009), due to factors such as paternal leakage into the mitogenome (e.g., Fontaine et al. 2007; Mastrantonio et al. 2019) and the presence of mitochondrial pseudogenes (Song et al. 2008; Buhay 2009). We followed recommendations by Buhay (2009) and found no evidence of pseudogenes (i.e., premature stop codons and presence of indels) in our *O. sheltae* *CO1* sequences. We did detect the presence of putative pseudogenes in some *O. australis* sequences, however, and these sequences were excluded from analyses.

Conclusions and recommendations

In this study, we reported on the first observations of *O. sheltae* at Shelta Cave since 1988. The rediscovery of this single-cave endemic crayfish offers optimism that other cave and groundwater species that have not been observed in several decades may still persist but remain at high risk of extinction. In the case of *O. sheltae* and the stygobiotic life at Shelta Cave in general, visual counts during recent surveys remain just a fraction of abundance observed over half a century ago. We generated the first genetic data and conducted the first phylogenetic analysis of *O. sheltae* finding strong support for placement of this species in the genus *Cambarus* with several other cave-obligate *Cambarus* species that occur in northern Alabama.

We offer several recommendations for management, conservation, and future research of *O. sheltae*. First, we propose the establishment of a long-term monitoring program for *O. sheltae* and other stygobiotic life at Shelta Cave to assess trends over time. Our study employed visual encounter surveys that can only be conducted when water levels are low. The use of baited funnel traps may be advantageous to increase detection for stygobiotic crayfishes (Crandall 2016; Fenolio et al. 2017; DiStefano et al. 2020), particularly during periods of high water. The use of environmental DNA (eDNA) for detecting and monitoring groundwater organisms has become increasingly popular in recent years. This approach, which leverages DNA shed by organisms

into their surrounding environment, has been employed with success in caves, springs, and wells for a diversity of groundwater organisms, including salamanders (Goricki et al. 2016, 2017; Voros et al. 2017; Lyons 2019), cavefishes (Lyons 2019; Mouser 2019; White et al. 2020; Mouser et al. 2021), amphipods (Niemiller et al. 2018), and crayfishes (Boyd 2019; Mouser 2019; Boyd et al. 2020; DiStefano et al. 2020; Dooley 2021; Mouser et al. 2021). With the first genetic data and tissues now available for *O. sheltae*, we recommend that a species-specific eDNA assay be developed and an eDNA survey study employed to potentially locate additional sites for this imperiled stygobiotic crayfish. Finally, new water quality analyses are needed to ascertain if contaminants, particularly pesticides and heavy metals, continue to percolate into the groundwater and determine if new threats exist that may impact the survival of the stygobiotic community at Shelta Cave.

Acknowledgements

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Supplementary material I

Table S1

Authors: Katherine E. Dooley, K. Denise Kendall Niemiller, Nathaniel Sturm, Matthew L. Niemiller

Data type: genetic sequences (docx. file)

Explanation note: **Table S1.** Genbank sequences included in the 16S and CO1 phylogenetic analyses.

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Link: <https://doi.org/10.3897/subtbiol.43.79993.suppl1>

Supplementary material 2

Table S2

Authors: Katherine E. Dooley, K. Denise Kendall Niemiller, Nathaniel Sturm, Matthew L. Niemiller

Data type: occurrence data (docx. file)

Explanation note: **Table S2.** Count data of cave crayfishes at Shelta Cave, Madison Co., Alabama from 1968 to 2021.

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A reassessment of the origin and distribution of the subterranean genus *Pseudolimnocythere* Klie, 1938 (Ostracoda, Loxoconchidae), with description of two new species from Italy

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Abstract

Groundwater ecosystems host a rich and unique, but still largely unexplored and undescribed, biodiversity. Several lineages of ostracod crustaceans have subterranean representatives or are exclusively living in groundwaters. The stygobitic genus *Pseudolimnocythere* Klie, 1938 has a West Palearctic distribution, and includes few living and fossil species of marine origin. Through a comprehensive literature review and the description of the two new living species, *Pseudolimnocythere abdita* **sp. nov.** and *Pseudolimnocythere sofiae* **sp. nov.**, from springs in the Northern Apennines, Italy, a morphological analysis was carried out with the aim of comparing the valve morphology of living and fossil species, and to discuss previous hypotheses about time and mode of colonization of inland waters. *Pseudolimnocythere* species show a low variability in valve morphology, with a remarkable stasis over geological times. The distribution of extant and fossil species is consistent with a scenario of multiple and independent events of colonization of continental habitats linked to sea level variations starting from Middle Miocene in the Paratethys and, later, in the Mediterranean. The most common colonization routes of inland waters have taken place through karst formations along ancient coastlines, although we cannot exclude some minor active migration through the hyporheic zone of streams. Available distribution data suggest a poor dispersal ability of *Pseudolimnocythere* species after they had colonized continental waters.

Keywords

colonization, evolution, stygobitic, morphology, taxonomy

Introduction

Although groundwater ecosystems form the largest freshwater biome (Griebler et al. 2014), they belong to the least explored habitats on earth (Ficetola et al. 2019). Many taxonomic lineages have a high biodiversity and are often represented by several endemic or rare species in groundwater (Bregović et al. 2019; Bishop et al. 2020), but their actual species richness, distribution patterns, and the ecological determinants shaping their areals are still poorly known (Deharveng et al. 2009; Stoch and Galassi 2010; Eme et al. 2015; Zgmajer et al. 2018). Ninety-five percent of global freshwater (excluding the polar ice caps) is stored in the continental subsurface constituting a major source of ecosystem services (Griebler et al. 2014). However, at the same time, subterranean ecosystems are threatened by different anthropogenic impacts and by a general inadequacy of protection policies, conditions that seriously endanger their biological diversity and the ecosystem services they provide (Boulton et al. 2008; Mammola et al. 2019). Improving our knowledge of groundwater fauna and its origin is thus of paramount importance for implementing effective conservation practices.

Crustacea is by far the most diversified stygobitic taxon in Europe, contributing to about 70% of the overall groundwater species richness (Zgmajer et al. 2018). The study of crustacean fauna has been especially important in developing models of colonization of continental subterranean waters (Stoch and Galassi 2010; Bauzà-Ribot et al. 2012; Delić et al. 2020). In particular, the class Ostracoda has been used to investigate time and mode of evolutionary radiation and colonization patterns (Danielopol 1980; Danielopol et al. 1994; Horne 2003).

It is well documented that stygobionts (i.e., taxa occurring exclusively in groundwater during their entire life cycle) can have a double origin (Coineau and Boutin 1992): limnicoid stygobionts derive from epigeal freshwater ancestors, while thalassoid stygobionts derive directly from marine ancestors. In the last case, pre-adapted marine species (Iglikowska and Pawłowska 2015), living in the interstitial or in fissured rocky habitats, could have crossed the salinity boundary via an intermediate mixo- to oligohaline coastal zone, and eventually have migrated in inland groundwaters (Boutin and Coineau 1990; Noteboom 1991). It has been suggested that between 9 and 12 independent invasions of freshwaters by marine ostracods have occurred, the first possibly in the early Carboniferous (Iglikowska 2014; Iglikowska and Pawłowska 2015).

The class Ostracoda consists of bivalved crustaceans with a laterally compressed body. Their calcitic carapaces are the most abundantly preserved arthropod remains in the fossil record (Matzke-Karasz and Smith 2020). Ostracods occur in almost all aquatic and in some semi-terrestrial habitats (Smith et al. 2015). Following Meisch et al. (2019), freshwaters host 2,330 accepted species of ostracods assigned to 270 genera of the order Pocodocopida, living both in surface than subterranean waters. In recent years, several papers reported the

description of the poorly known subterranean ostracod faunas from different parts of the world (e.g., Karanovic 2007; Reeves et al. 2007; Smith 2011; Peterson et al. 2013; Iepure et al. 2016; Külköylüoğlu et al. 2017; Mazzini et al. 2017; Pociecha et al. 2021).

The ostracod family Loxoconchidae has living representatives in marine, brackish and freshwater habitats (Athersuch and Horne 1984). Recent non-marine Loxoconchidae have a Palearctic distribution; ten species in five genera are currently known, accounting for c. 0.4% of the total freshwater ostracod diversity (Meisch et al. 2019). Within Loxoconchidae, the stygobitic genus *Pseudolimnocythere* Klie, 1938 has a West Palearctic distribution, and includes few Recent and fossil freshwater species of marine origin. Danielopol (1980) argued that the genus *Pseudolimnocythere* was closely related to the marine interstitial genus *Tuberoloxoconcha* Hartmann 1974 (the most notable difference between the species of the two genera is the length of the antennular distal article), the latter genus placed in the subfamily Pseudolimnocytherinae Hartmann and Puri 1974 within the Loxoconchidae. Danielopol (1979) accommodated the genus *Pseudolimnocythere* within the tribe Pseudolimnocytherini. The subfamily Pseudolimnocytherinae and the tribe Pseudolimnocytherini are no longer recognized as valid after the results of a cladistic analysis, based on morphocharacters of Recent freshwater ostracods, which showed that *Pseudolimnocythere* and *Loxoconcha* Sars, 1866 form a more derived clade within the family Loxoconchidae (Savatenalinton and Martens 2009). Besides its taxonomic interest, the genus *Pseudolimnocythere* has been the subject of several studies aimed at investigating the evolution of subterranean ostracods and their colonization modes (Danielopol 1977, 1979, 1980; Danielopol and Bonaduce 1990).

In this paper, after a comprehensive literature review and the description of two new living species from springs in the Northern Apennines, Italy, we critically revise the previously proposed scenarios in the light of the new available data and offer a morphological analysis of all living and fossil *Pseudolimnocythere* species known so far. After the taxonomic analysis, we used morphological and biogeographic tools to elucidate the origin and colonization of continental waters by this genus. We tested two different hypotheses: (i) a “single marine invasion” where pseudolimnocytherids obtained their current distribution through dispersal, mainly in interstitial habitats; (ii) a “multiple marine invasion” where a previous dispersal along marine coasts is followed by a very limited dispersal in freshwaters and a long history of speciation events due to vicariance.

Materials and methods

Ostracod specimens described in this paper were sampled from the Cirone rheocene spring and the Poiano spring system (Emilia Romagna region) (Suppl. material 1: Fig. S1). In the Cirone spring, a baited trap was used, as described in Bottazzi et al. (2011). In the Poiano spring system, a drift net was positioned to filter the entire discharge of the major spring mouth.

Ostracods were preserved in ethanol 75%. Dissections were done under a stereomicroscope (Zeiss 47 50 22). Valves were examined by Scanning Electron Microscopy (SEM) using a Philips XL-30 and a FEI Quanta 400, and then stored dry in micropaleontological

slides; soft parts were dissected in glycerine, mounted in glycerine on a microscope glass slide and sealed using nail polish. Drawings of soft parts were made with the aid of a camera lucida attached to a compound microscope (Zeiss 47 30 11-9901).

Chaetotaxy of the limbs in descriptions follows Meisch (2000), and higher taxonomy of the Ostracoda is according to Meisch et al. (2019).

The outlines of valves obtained both from SEM images and drawings taken from literature, were gathered using GIMP (GNU Image Manipulation Program, <https://www.gimp.org>), then saved as TPS file using the TPSutil32 v. 1.76 software and digitized using the TPSdig v. 2.20 software (Rohlf 2009). Superimposition of valve outlines was performed using Morphomatica 1.6.0.1 (Linhart et al. 2007), a program designed for the approximation of ostracod specimens using an adapted B-splines algorithm. For the approximation method in Morphomatica, 16 arbitrary control points were considered with 6 iteration steps.

Results

Taxonomic account

Class Ostracoda Latreille, 1802

Subclass Podocopa Sars, 1866

Suborder Cypridocopina Baird, 1845

Order Podocopida Sars, 1866

Superfamily Cytheroidea Baird, 1850

Family Loxoconchidae Sars, 1925

Genus *Pseudolimnocythere* Klie, 1938

Diagnosis (modified from Danielopol 1979 and Karanovic 2012). Small ostracods (length < 0.4 mm), without sexual dimorphism. Carapace ornamented with rounded pits. Hinge henodont (single posterior tooth in RV), invicidont, or amphidont. Calcified inner lamella wide, marginal pore canals branched; normal pore canals with small sieve plates located in deep funnels. Antennula, six-segmented (fourth and fifth segments only partially separated or completely indistinct), the distal segment exceeding the length of the second segment. Exopod of antenna not segmented; penultimate segment with two basal setae and one aesthetasc, and two distal (anterior) setae.

***Pseudolimnocythere abdita* sp. nov.**

<http://zoobank.org/6EA43D77-BD52-461F-B5CF-266AD34C9E79>

Figs 1, 2, 3A, B

Type locality. Rheocrene spring Cirone, road from Bosco di Corniglio to Cirone pass, municipality of Corniglio, province of Parma, Emilia-Romagna region, coordinates

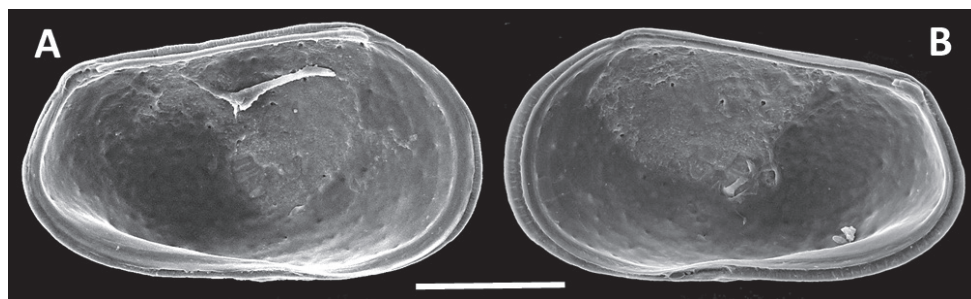


Figure 1. *Pseudolimnocythere abdita* sp. nov., VP1152, adult ♀ **A** left valve, internal view **B** right valve, internal view. Scale bar: 100 μm .

44°26'59"N, 10°00'38"E, 1126 m a.s.l. (Suppl. material 1: Figs S1, S4A). Natural free-flowing spring located on shales and limestone (Suppl. material 1: Fig. S2), estimated average discharge 2 l s⁻¹, water temperature 7.9–8.5 °C, electric conductivity c. 360 $\mu\text{S cm}^{-1}$, pH 7.5–7.7 (Bottazzi et al. 2011). Sample collected by Elisa Bottazzi on May 15, 2007.

Material investigated. One adult female designated as holotype, with valves stored dry in a micropaleontological slide (used for SEM) and soft parts dissected in glycerine and kept in a sealed slide (VP1152). Previously reported as *Pseudolimnocythere* cf. *hypogea* Klie, 1938 in Bottazzi et al. (2008, 2011) and *P.* sp. 1 in Pieri et al. (2015). Material deposited in the Ostracod Collection of the Department of Chemistry, Life Sciences and Environmental Sustainability, University of Parma.

Derivation of name. From the Latin adjective *abditus*-a-um, meaning hidden, concealed, but also mysterious, due to the fact that only one individual of this species was collected despite repeated samplings in the type locality and in other springs of the same region.

Description. Valves (Figs 1, 3A, B). Subrectangular in lateral view. Anterior margin broadly rounded, posterior margin rounded and blunty pointed. Surface ornate with fossae. Hinge reduced invicident: LV with smooth bar, smooth anterior tooth and tripartite posterior socket; RV with smooth groove and anterior socket, posterior tooth consisting of a large element and two smaller teeth towards the anterior part. Muscle scars not visible.

Appendages (Fig. 2). Antennula six-segmented. Second podomere with setulae on anterior margin and a ventro-apical seta slightly longer than the next segment; third podomere short, bearing a dorso-apical seta about two times its length; fourth segment formed by fourth and fifth podomeres partially fused, with one posterior and one anterior subequal setae inserted near fusion line, and distally one posterior seta and three anterior setae reaching or slightly beyond tip of next segment; terminal podomere long and thin, distally with a seta fused at the base with aesthetasc y_a , and a free seta of the same length as the latter. Antenna with robust basipodite. Spinneret seta (exopodite) gently arched proximally, central part nearly straight, distal end decidedly bent and with pointed tip almost reaching distal end of terminal

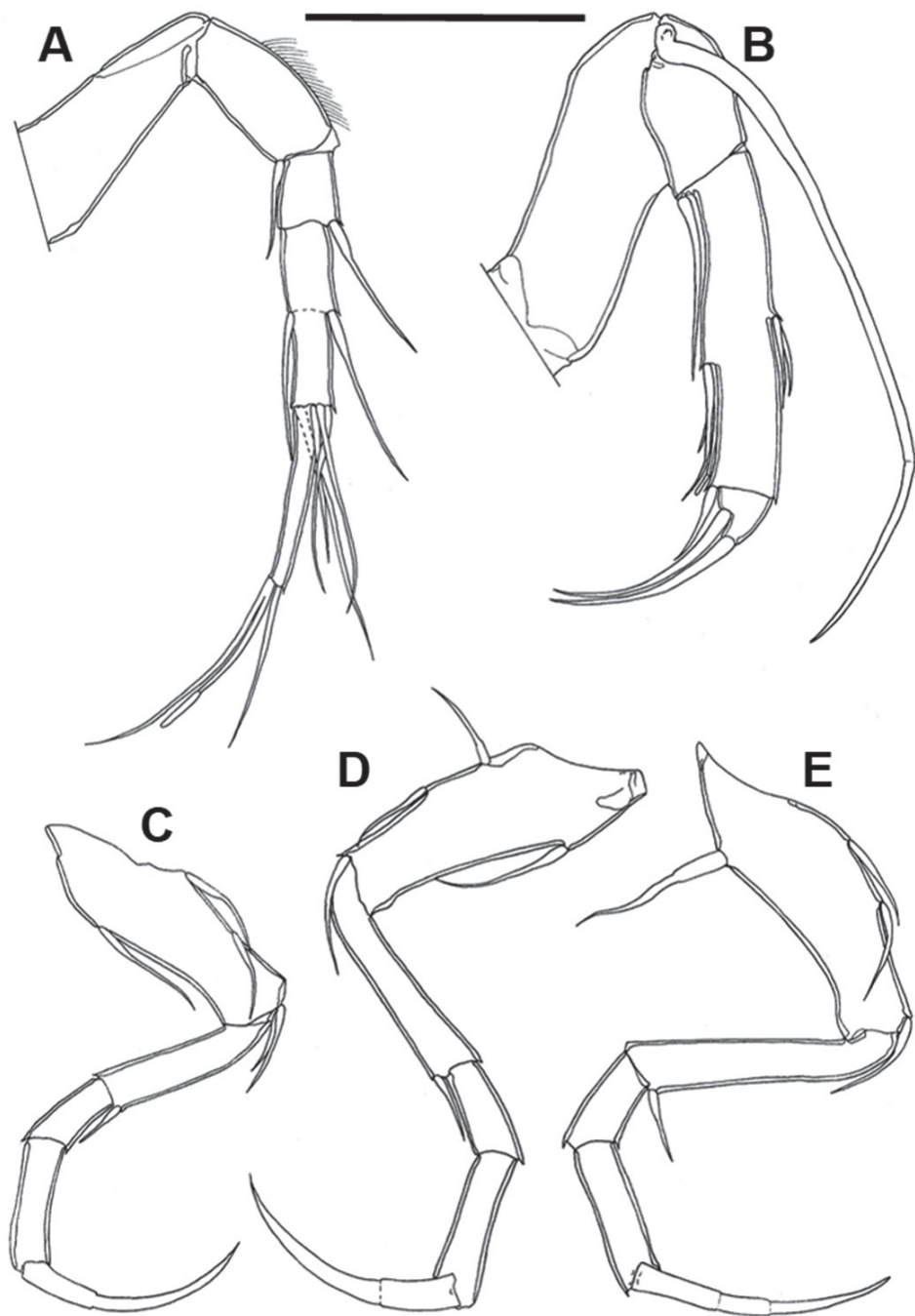


Figure 2. *Pseudolimnocythere abdita* sp. nov., VP1152, adult ♀ **A** antennula **B** antenna **C** first thoracopod **D** second thoracopod **E** third thoracopod. Scale bar: 50 μ m.

claws. Endopodite four-segmented. First podomere with a ventro-apical setae reaching slightly beyond mid-length of next segment. Penultimate segment consisting of second and third segments fully fused, with two short setae inserted halfway the length of anterior margin, and two unequal setae and aesthetasc Y at c. 4/7 of posterior margin, tip of the latter slightly surpassing segment distal end, and one robust ventro-apical seta; last segment with two claws, one longer inserted more proximally and the other apically. Thoracopods (walking legs) four-segmented, first podomere sturdier and more enlarged than following ones. First segment of first thoracopod with four setae in anterior position, one proximally, one medially and two distally, and a posterior seta inserted proximally; second podomere with a dorso-apical seta shorter than next segment; third and fourth segment without setae; last podomere distally bearing a robust claw with proximal third enlarged. Second and third thoracopod with analogous setal arrangement; first segment with three anterior setae and a longer posterior seta; second segment with a dorso-apical seta, third segment without setae; terminal claw of second thoracopod more enlarged and stouter than in other thoracopods, that of third thoracopod consisting of three parts with gradually decreasing diameter. Male unknown.

Measurements. Valve length 308 μm , height 173 μm ($n = 1$).

Distribution. The species is known from its type locality only.

Differential diagnosis. *Pseudolimnocythere abdita* sp. nov. differs from other described living or subfossil species of the genus with “sloping” valves (see below) in its intermediate size, being larger than *P. sp.* sensu Schornikov, 2013 (c. 0.25 mm) and *P. sp.* Montanari et al. 2021 (c. 0.28 mm), and shorter than *P. sp.* Peterson et al. 2013 (c. 0.35 mm), and *P. sp.* Danielopol 1980 (0.34–0.36 mm). For all these species, only the morphology of the valves is known, therefore a comparison with the soft parts of *P. abdita* sp. nov. is not possible.

Remarks. *Pseudolimnocythere abdita* sp. nov. is here formally described, in spite of the fact that a single female specimen was available. We have decided to do this for the following reasons:

- both valve and soft features are described, allowing us to clearly distinguish the new species from its congeners;
- so far only two other living species are known for the genus *Pseudolimnocythere*, which is of particular importance to better understand the origin, phylogenetic affinities and distribution of non-marine representatives of the family Loxoconchidae;
- the habitat from which the species was collected strikingly differs from those of other living congeners (see below);
- further samplings performed in the type locality and surrounding spring areas did not yield additional specimens.

We are thus confident that *Pseudolimnocythere abdita* sp. nov. can be unambiguously identified on the characters presented here.

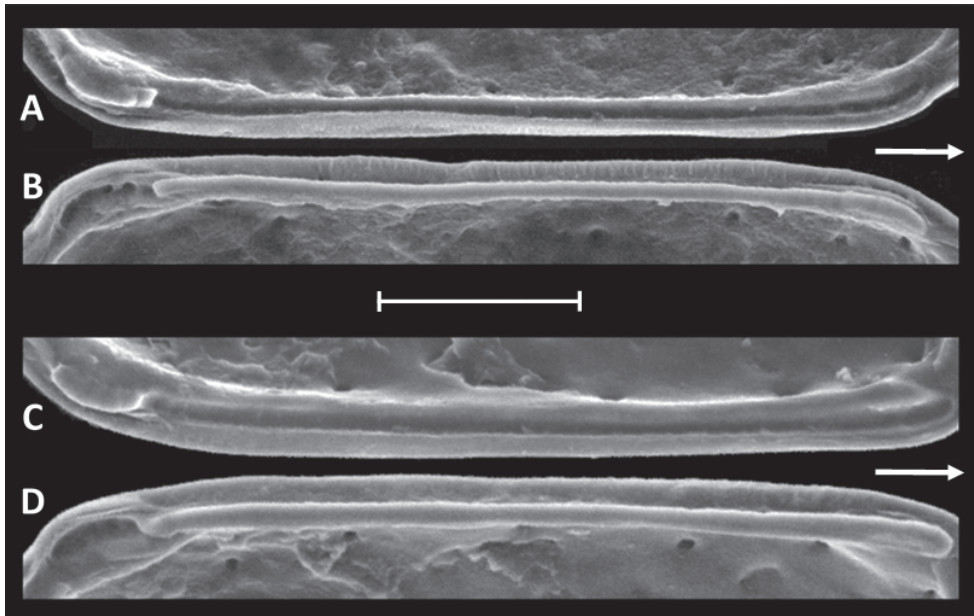


Figure 3. **A, B** *Pseudolimnocythere abdita* sp. nov., VP1152, adult ♀ **C, D** *Pseudolimnocythere sofiae* sp. nov., VP1125, adult ♂ **A** right valve, internal view, detail dorsal margin **B** left valve, internal view, detail dorsal margin **D** right valve, internal view, detail dorsal margin **E** left valve, internal view, detail dorsal margin. Scale bar: 40 μm.

***Pseudolimnocythere sofiae* sp. nov.**

<http://zoobank.org/060FAE40-34C2-45BE-885F-D26F96B8ADF8>

Figs 3C, D, 4, 5

Type locality. Poiano springs, Upper Secchia Valley, municipality of Villa Minozzo, province of Reggio nell'Emilia, Emilia-Romagna region, coordinates 44°23'31"N, 10°26'20"E, 423 m a.s.l. (Suppl. material 1: Figs S1, S4B). Average discharge > 400 l s⁻¹, water temperature between 8.9 and 10.8 °C, conductivity ranging from ~9 to ~17 mS cm⁻¹ (Stoch et al. 2009a). Poiano is the major spring complex of a large outcrop of Upper Triassic evaporites, comprising a sequence of gypsum-anhydrite and dolostone beds with local salt bodies (Suppl. material 1: Fig. S3). The spring drains an aquifer of unique properties composed of anhydrite with halite lenses at depth and gypsum at the surface (both with high NaCl content) (Chiesi et al. 2010). Sample collected by Fabio Stoch on December 21, 2015.

Material investigated. More than 30 specimens from the type locality and sample of the holotype, and additional specimens from nearby localities (see below). Previously reported as *Pseudolimnocythere* sp. in Stoch et al. (2009a, b) and *P.* sp. 1 in Pieri et al. (2015).

Holotype. GR804, adult ♂, soft parts dissected in glycerine in a sealed slide, valves used for SEM imaging and stored dry in a micropalaentological slide.

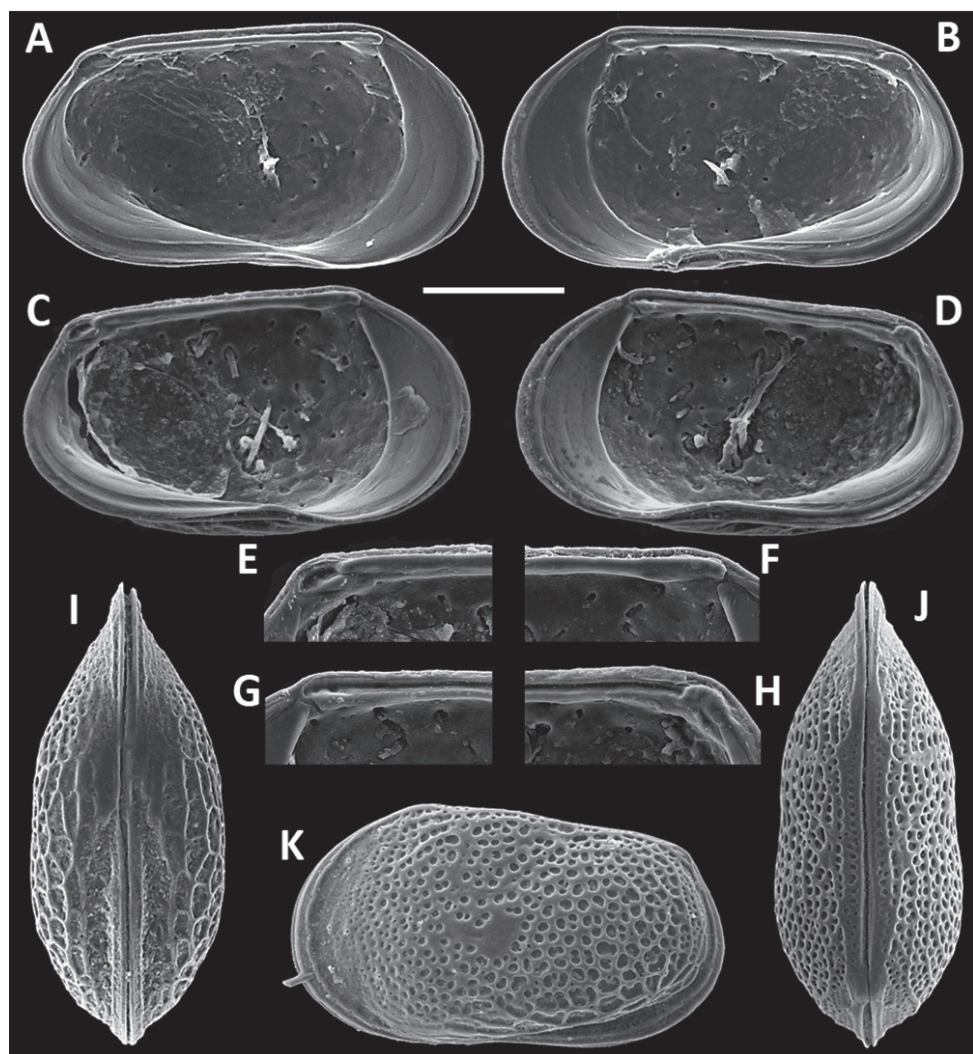


Figure 4. *Pseudolimnocythere sofiae* sp. nov. **A, B** VP1125, adult ♂ **C–H** GR797, adult ♀ **I** VP1120, adult (sex undetermined) **J** VP1122, adult (sex undetermined) **K** VP1121, adult (sex undetermined) **A** left valve, internal view **B** right valve, internal view **C** left valve, internal view **D** right valve, internal view **E** left valve, internal view, detail postero-ventral corner **F** left valve, internal view, detail antero-dorsal corner **G** right valve, internal view, detail antero-dorsal corner **H** right valve, internal view, detail postero-ventral corner **I** carapace, dorsal view **J** carapace, ventral view **K** carapace, left lateral view. Scale bar: 100 µm (**A–D, I–K**); 66 µm (**E–H**).

Derivation of name. This species is dedicated to GR's daughter, Sofia Rossetti Tekleab. Furthermore, among the different meanings of the ancient Greek noun "σοφία" there are also "knowledge" and "wisdom". Our hope is that the description of this new species will shed more light on morphological characteristics and evolutionary relationships of the genus *Pseudolimnocythere*.

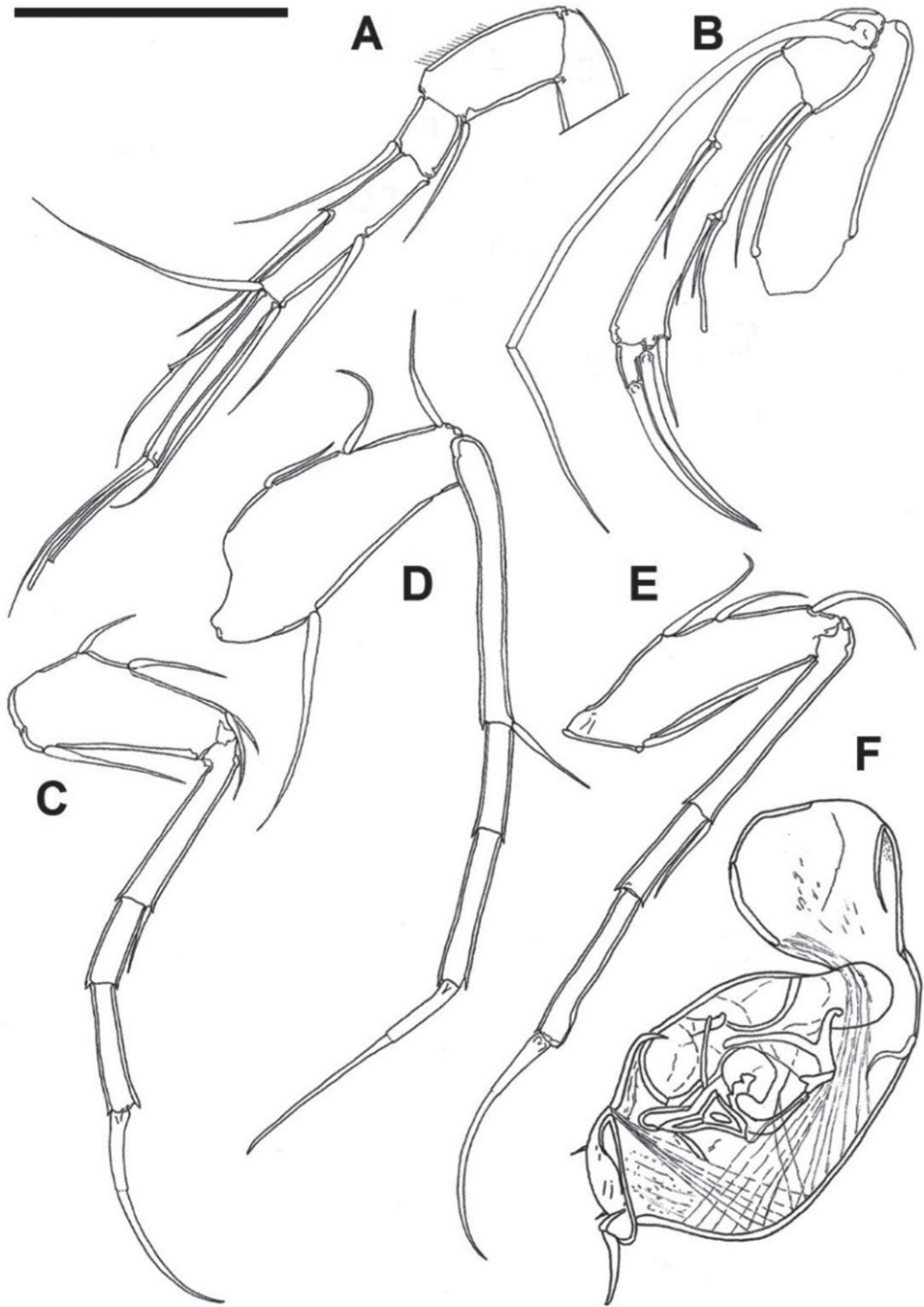


Figure 5. *Pseudolimnocythere sofiae* sp. nov., GR804, adult ♂ **A** antennula **B** antenna **C** first thoracopod **D** second thoracopod **E** third thoracopod **D** hemipenis. Scale bar: 50 μ m (**A**, **E**); 64 μ m (**F**).

Description. Valves (Figs 3C, D, 4). Carapace small, in dorsal view elliptical, valves sub-rectangular in lateral view. Viewed laterally the dorsal edge is sloping gently posteriorly turning into a curve. Anterior end of the carapace broad and rounded. Maximum length at mid height, maximum height in the anterior third. Surface ornamented with subrounded pits, with dimensions increasing towards posterior. In dorsal view, anterior end beak shaped. In internal view: vestibulum well developed, selvage strong and narrow, line of concrescence well developed. Dorsal margin straight, ventral margin concave in the middle part, with an additional closing mechanism where the RV overlaps the LV. Hinge amphidont: LV with smooth cardinal bar swelling anteriorly into a bi or trilobate tooth, posteriorly with a lower small tooth and an upper large socket with several lobes, RV with complementary intercardinal groove, swelling anteriorly into two-three sockets, posterior element with a multilobate tooth. Muscle scars typical of the family: four adductor muscle scars in a vertical row. Sexual dimorphism not very pronounced, with males slightly larger and stouter than females. Average carapace length 0.32 mm.

Appendages (Fig. 5). Antennula slender, six-segmented. Second podomere with short setulae on the second half of the anterior margin and a ventro-apical seta reaching c. $1/3$ of the length of next segment; third podomere short, with a dorso-apical seta slightly shorter than next segment; penultimate segment consisting of fourth and fifth podomeres fused, with two subequal setae, one posterior and one anterior, at junction of the two fused segments, and distally one posterior seta and three anterior setae, the longer ones reaching beyond tip of next segment; terminal podomere long and thin, distally with a free seta and aesthetasc y_a fused at the base with a seta. Antenna with stout, trapezoidal basipodite. Spinneret seta (exopodite) reaching tip of distal claws of endopodite, proximally bowed and nearly straight in the middle part, distal end folded and thin. Endopodite four-segmented; first segment of endopodite with a ventro-apical setae slightly exceeding mid-length of next segment; penultimate segment formed by second and third podomeres fused, with two setae of different length at c. $1/3$ of anterior margin, aesthetasc Y and two shorter setae at about half length of posterior margin, and one ventro-apical seta; last segment with two claws, the longest one proximo-posterior and the other distal. Thoracopods (walking legs) four-segmented, with first podomere stout and remaining ones long and slender with straight margins. First segment of first thoracopod with four anterior setae, one proximal, two median and one distal, and a proximo-posterior seta reaching the end of the segment; second podomere with a dorso-apical seta; third and fourth segment without setae; last segment with a distal claw weakly subdivided in two parts, the distal one c. $2/3$ of the length and narrower. Same setal formula in second and third thoracopod: first segment with three anterior setae and a longer posterior seta; second segment with dorso-apical seta; third segment with no setae; last segment with long claws. Hemipenis with chitinized anterior lobe having a narrow base and distally wider and sub-quadrate.

Measurements. Valve length 303–331 μm ($n = 9$).

Distribution. In addition to individuals drifted from the Poiano spring, rare specimens of *Pseudolimnocythere sofiae* sp. nov. were found in some nearby habitats reported by Stoch et al. (2009b): in the interstitial of Secchia river (where spring waters come

out), Sologno stream and Lucola stream; in the gypsum caves named Risorgente di Ca' della Ghiaia (cadastral number 244 ER) and Tanone Grande della Gaggiolina (cadastral number 154 ER); no specimens were found in all other types of groundwater and interstitial habitats examined in the same area, or in springs located in soils on marly-arenaceous deposits upstream of the evaporite outcrops (Stoch et al. 2007, 2009b).

Differential diagnosis. *Pseudolimnocythere sofiae* sp. nov. is easily distinguishable from described congeneric species. The better described species is *P. hartmanni* Danielopol (1979) from which it differs in overall outline, size, ornamentation and development of the ventral posterior margin. *Pseudolimnocythere* sp. (Peterson et al., 2013, fig. 8U) seems much larger, although measurements were taken from the figure (where a LV is erroneously reported as a RV). *Pseudolimnocythere hypogaea* (sensu Karanovic and Pesce 2001, figs 1–6) is slightly smaller, stouter in the overall appearance and with a characteristic wavy dorsal margin. *Pseudolimnocythere* sp. (Danielopol 1980, fig. 11A–F) from the Skulijca cave displays a faint ornamentation and a stout anterior marginal rim; the overall external shape is not visible in the photos provided but in internal view the central curve of the ventral margin is less pronounced.

Pseudolimnocythere sp. (Peterson et al. 2013) is stouter, with a different surface ornamentation and an oblique dorsal margin. *Pseudolimnocythere* sp. sensu (Schornikov et al., 2014) is slightly smaller (302–311 µm), with a surface ornamentation occurring only in the marginal areas. The specimen illustrated in Schornikov et al. (fig. 4k, 2014) is probably a juvenile. The drawings of *P. hypogaea* by Klie (1938) and Karanovic and Pesce (2001) clearly display a sinuous dorsal margin and the lack of the additional closing mechanism in the ventral area. The morphology of the hemipenis differs markedly from that of the extant species of *Pseudolimnocythere* for which males have been described (Fig. 6).

Note. The distal segment of walking legs in *Pseudolimnocythere abdita* sp. nov. and *P. sofiae* sp. nov. is fused with the basal part of the claws (Figs 2C–E, 5C–E). The same pattern is reported for the Limnocytheridae as well (see Meisch 2000).

Other records of living and sub-Recent *Pseudolimnocythere*

Pseudolimnocythere sp. (fig. 8U in Peterson et al. 2013)

Frasassi cave complex (Marche, Italy), inner lakes of the Grotta del Fiume (cadastre number 8 Ma), in the remains of the subfossil eels at Lago delle Anguille; outside the cave, sulfidic springs on the bank of Sentino River (Fig. 9C). More than one thousand subfossil carapaces and valves recovered from various sites in the cave, possibly dating back 7,200 years (Mariani et al. 2007). Previously reported as *Pseudolimnocythere* cf. *P. hypogaea* by Peterson et al. (2009).

Pseudolimnocythere hypogaea Klie 1938

Slightly brackish groundwater habitats in Apulia, Italy, were cited as 'type localities' by Klie (1938): wells near Bari (Fig. 9E) and in two caves, L'Abisso (cadastre number 141



Figure 6. Hemipenis of Recent species of *Pseudolimnocythere* **A** *P. hypogaea* (redrawn from Klie, 1938) **B** *P. hypogaea* (redrawn from Karanovic and Pesce 2001) **C** *P. hartmanni* (redrawn from Danielopol 1979). Not to scale.

Pu, Castro Marina, Lecce) and Cunicolo dei Diavoli (101 Pu, Porto Badisco, Lecce), these latter located c. 10 km apart (Fig. 9F). Later found again (reported as *P. hypogaea*) in L'Abisso cave, and in a well on the road Foggia-Manfredonia, Apulia (site PU56) (Fig. 9G) by Karanovic and Pesce (2001), who also gave a redescription of this species. L'Abisso cave is located a few dozen metres from the sea and its water is brackish, the salinity varying in relation to the seasonal mixing of seawater and groundwater (Inguscio et al. 2009). PU56 is defined as a “freshwater well” located on the Gargano promontory at about 10 km from the coast, but actually this area is characterised by a karst aquifer with brackish waters (FS, pers. obs.). Finally, the cave named Cunicolo dei Diavoli opens at the seashore, it is flooded by brackish as well as marine waters (being influenced by the effect of the tides). Karanovic and Pesce (2001, pages 22 and 23) remarked some differences in valve size and chaetotaxy with respects to the description of *P. hypogaea* by Klie (1938). Since in both cases the descriptions were made on material collected from different localities, the existence of different species, albeit strongly related to each other, cannot be excluded. Differences in valve size and outline between specimens illustrated by Klie (1938) and Karanovic and Pesce (2001) can be observed in Fig. 7C, D.

***Pseudolimnocythere* sp. (Danielopol and Bonaduce 1990, not figured)**

Interstitial of a temporary stream, beach in front of La Baillaury (site Ba-2), Bay of Banyuls, France (Fig. 9A). The salinity of the interstitial waters varies according to the stream flow (14‰ in May 1984). A single adult male, not figured.

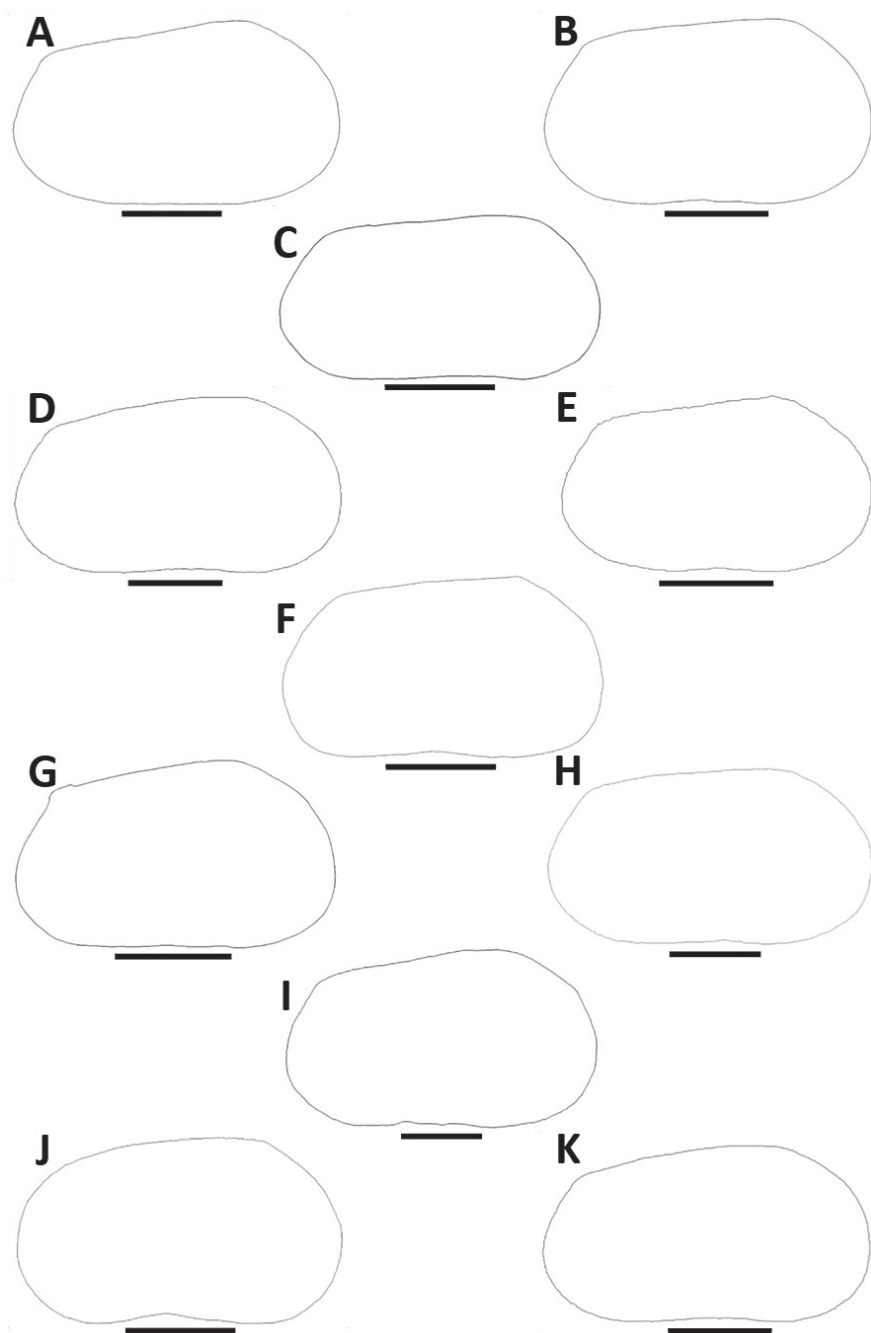


Figure 7. Valve outlines of Recent and fossil species of *Pseudolimnocythere* **A** *P. Pseudolimnocythere abdita* sp. nov. **B** *P. sofiae* sp. nov. **C** *P. hypogaea* (from Klie 1938) **D** *P. hypogaea* (from Karanovic and Pesce 2001) **E** *P.* sp. (from Peterson et al. 2013) **F** *P. hartmanni* (from Danielopol 1979) **G** *P.* sp. (from Danielopol 1980) **H** *P.* sp. (from Montanari et al. 2021) **I** *P.* sp. sensu Schornikov 2013 (from Schornikov et al. 2014) **J** *P. hainburgensis* (from Danielopol et al. 1991) **K** *P. hainburgensis* (from Gross and Piller 2006). Scale bar: 100 μ m (in case of intraspecific variability in valve size, the maximum value is considered).

***Pseudolimnocythere* sp. (fig. 7G in Danielopol 1980)**

Cave Skuljica, Krk Island, Croatia (Fig. 9H). Only carapaces and valves, no living specimens.

Pseudolimnocythere hartmanni (fig. 3 in Danielopol 1979 and fig. 10A–H in Danielopol 1980)

A well fed by freshwater, 19 m from the seashore on the northwestern coast of Euboea (Evvia) Island, village Aghios Georghios, Greece (Fig. 9J).

***Pseudolimnocythere* sp. sensu Schornikov 2013 (Ivanova et al. 2014; figs 4I–N in Schornikov et al. 2014)**

Inner shelf of the northeastern coast of Black Sea, approximately 2.4 km from the town of Gelendzhik, and living in the freshwater underflow of the small Jeane River, 16 km upstream off the town of Gelendzhik (Fig. 7K). This species was first recorded as *Elofsonia*? sp. (Schornikov 2012).

?*Pseudolimnocythere* sp. A (figs 4o, p in Schornikov et al. 2014)

Black Sea, Tsemes Bay (town of Novorossiysk) at a depth of 2 m. The attribution of the figured specimens to the genus *Pseudolimnocythere* is doubtful and therefore no further considered herein.

***Pseudolimnocythere* sp. (Montanari et al. 2017, 2021)**

Vodeni Rat anchialine cave, 13 m above sea level, and about 30 m inland from the rocky coast, Sveti Klement Island, Pakleni Archipelago, southeastern of the island of Hvar (Croatia) (Fig. 9I). Karstic well with freshwater of meteoric origin until the 4th–7th century CE; today flooded by anchihaline waters, with a stratification with marine waters at the bottom (Montanari et al. 2021). Twenty-five well-preserved single valves, no soft parts nor paired and closed articulated valves.

Remarks

The described species of the genus *Pseudolimnocythere* show low variability in valve morphology, and for extant species the identification is mainly based on male copulatory organ morphology. This situation makes it difficult to identify and compare both fossil and Recent species exclusively through the morphological analysis of valves, or when male sexual characters cannot be examined in living species. Therefore, definite criteria for species assignment which are valid both in neontological and paleontological research are presently unavailable for this genus. Nevertheless, analysis of valve outlines in normalized area mode allowed to identify three distinct morphological groups. The first group, characterized by “curved” valves (rounded postero-dorsal margin, dorsal margin gently arched, convex ventral margin),

contains *P. hainburgensis* only; the second group, consisting of species with “sloping” valves (dorsal margin straight and decidedly sloping backward, in some species also a straight postero-dorsal margin), includes *P. abdita* sp. nov., *P. sp.* Peterson et al., 2013, *P. sp.* sensu Schornikov, 2013, *P. sp.* Danielopol, 1980 and *P. sp.* Montanari et al., 2021; the third group joins species with “slightly inclined” valves (dorsal margin straight, but less inclined backwards than in the previous group), namely *P. sofiae* sp. nov., *P. hypogaea* and *P. hartmanni* (Fig. 8). The three groups are here used for convenience, and no taxonomic value should be attributed to them. We did not consider it useful to proceed with a statistical analysis of the valve outlines. In fact, despite the supposed absence of a significant sexual dimorphism in the valve shape and the symmetry between right and left valve in *Pseudolimnocythere*, the scarcity of figured specimens - and sometimes also their low quality - in the literature forced us to compare outlines obtained by individuals of different gender, and in some cases after reversal of the valves. It is worth emphasizing that the three Recent species whose morphology of the male copulatory organs is known (*P. hypogaea*, *P. hartmanni* and *P. sofiae* sp. nov.) and which certainly constitute distinct taxonomic entities (Fig. 6), are found in the same cluster defined by valve shape. However, this does not happen for the specimens of *P. hypogaea* described by Karanovic and Pesce (2001) for which, as mentioned above, there is some uncertainty about their identification with the material examined and figured by Klie (1938). Another potentially applicable criterion is that based on difference in adult valve size: at the lower extreme of the range are *P. sp.* sensu Schornikov, 2013 (c. 0.25 mm) and *P. sp.* Montanari et al., 2021 (c. 0.28 mm), while *P. sp.* Danielopol (1980) (0.34–0.36 mm) and *P. sp.* Peterson et al. (2013) (c. 0.35 μ m) define upper size limits; the remaining species have intermediate sizes (Fig. 7).

There are several differences in the morphology of the hinge among *Pseudolimnocythere* species. On the other hand, different types of these structures are known for the family Limnocytheridae, even at the genus level (Yamaguchi 2003, Savatnalinton and Martens 2009).

Records of fossil *Pseudolimnocythere*

Pseudolimnocythere hainburgensis (fig. 7I, J in Danielopol et al. 1991).

Miocene (Badenian) of the Vienna Basin, Hainburg, Lower Austria (Fig. 9L). Gross and Piller (2006) further recorded *P. hainburgensis* from the same formation.

According to Danielopol et al. (1991), *P. hainburgensis* probably lived in fresh- or brackish water habitats since it was associated with Characeae gyrogonites and the freshwater ostracods *Darwinula* and *Candona*. Although strongly resembling each other in valve morphology, there are some differences in size and valve outline between the specimens of *P. hainburgensis* described by Danielopol et al. (1991) and Gross and Piller (2006) (Figs 7, 8). The observed morphological variations can be ascribed either to intraspecific variability and/or occurrence of different species.

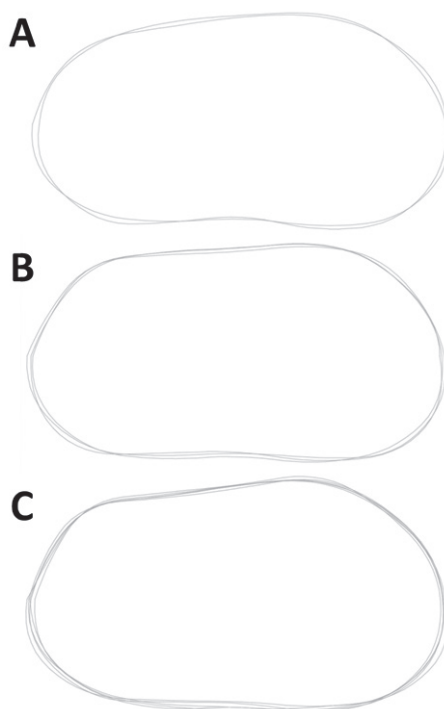


Figure 8. Superimposition of valve outlines of Recent and fossil species of *Pseudolimmocythere* in normalized area mode **A** “curved” valves: *P. hainburgensis* (from Danielopol et al. 1991 and Gross and Piller 2006) **B** “slightly inclined” valves: *P. sofiae* sp. nov., *P. hypogaea* (from Klie 1938), *P. hartmanni* (from Danielopol 1979) **C** “sloping” valves: *P. abdita* sp. nov., *P. sp.* (from Peterson et al. 2013), *P. sp.* (from Montanari et al. 2021), *P. sp.* sensu Schornikov, 2013 (from Schornikov et al. 2014); *P. sp.* (from Danielopol 1980).

***Pseudolimmocythere* sp. Nachite et al. 2003 (not figured)**

The authors report seven specimens from the late Pliocene Saïss basin, Douar section, Northern Morocco. Possibly the same as *Pseudolimmocythere* sp. A Bekkali & Nachite, 2003 (see below).

***Pseudolimmocythere* sp. A (plate III–8 in Bekkali and Nachite 2006)**

Late Pleistocene, Saïss plain, Douar section, in an old quarry of Douar El Mechmach on the road from Fès to Aïn Chkef Morocco. One left valve, length ~230 µm, either small-sized species or A-1 stage.

***Pseudolimmocythere hartmanni* Danielopol 1979**

Cores drilled in the harbour of Salerno, Tyrrhenian Sea, Italy. Considered as an allochthonous species. It was found in sample GS1 7.50, Holocene fine sands and rare



Figure 9. Paleogeographic map of the Mediterranean basin and Western Paratethys during Mid-Miocene (13 Ma, after Ron Blakey, Colorado Plateau Geosystems, Arizona, USA, <http://cpgeosystems.com>) and distribution of Recent (circles) and fossil (stars) species of *Pseudolimnocythere* **A** *P.* sp. (Danielopol and Bonaduce 1990) **B** *P. abdita* sp. nov. **C** *P. sofiae* sp. nov. **D** *P.* sp. (Peterson et al. 2013) **E** *P. hypogaea* (Karanovic and Pesce 2001) **F** and **G** *P. hypogaea* (Klie 1938) **H** *P.* sp. (Danielopol 1980) **I** *P.* sp. (Montanari et al. 2021) **J** *P. hartmanni* (Danielopol 1979) **K** *P.* sp. *sensu* Schornikov 2013 (Schornikov et al. 2014) **L** *P. hainburgensis* (Danielopol et al. 1991) **M** *P.* sp. (Bekkali and Nachite 2006) **N** *P. hartmanni* (*sensu* Aiello et al. 2020).

gravels of the unit US5 (in some levels of the unit US5 ceramic fragments of Roman age occur, and in the lower part is present a level dated at 7553–7411 cal BP), and in sample GS1 16.50, Pleistocene, older than the Campanian Ignimbrite super-eruption (~40 ka), possibly the substage MIS 5e (Aiello et al. 2020).

The left valves of *P. hartmanni* illustrated in the literature (fig. 3 in Danielopol 1979 and fig. 6.9 in Aiello et al. 2020) have very similar length (0.29–0.30 mm), but the length/height ratios are 1.96 and 1.75, respectively. So far, it is impossible to establish whether these differences fall within the range of variability of *P. hartmanni*, or rather the specimens belong to different species. Due to the deteriorated margins of the valve, the specimen of *P. hartmanni* from Aiello et al. (2020) was not used for the analysis of the valve outlines. The record from Salerno, if confirmed, greatly expands the geographic range of *P. hartmanni*, previously known only for Euboea Island, Greece, as a living species.

Discussion

Pseudolimnocythere shows a pre-adaptation to groundwater lifestyle

Speciation in subterranean habitats is commonly explained as the result of divergent selection in geographically isolated populations (Konec et al. 2015; Mammola et al. 2018). The role of evolutionary conservatism and convergence arising from the selective pressure of groundwater environments result in similarity in stygobitic species traits over large spatial scales (Bauzá-Ribot et al. 2011). Small size, trapezoidal, triangular, or streamlined carapace shape and lack of swimming bristles are highly conservative morphological traits in stygobitic ostracods (Marmonier et al. 1994, Dole-Olivier et al. 2000).

Differences between extant *Pseudolimnocythere* species are mostly apparent in the structure of the male copulatory organ, while differences in other soft parts and valve morphology are limited to minor details. All known species of the genus, both fossil and extant, show substantial uniformity in size, with lengths in the 0.25–0.36 mm range. This morphological trait represents a pre-adaptation in carapace shape and size suitable for life in non-marine interstitial and porous or fissured groundwater aquifers. Carapace features show a remarkable evolutionary conservatism over time. The presence of different kinds of hinges is an exception to this valve morphological uniformity. It has been suggested that a complication of the hinge structure in loxoconchids, compared to the amphidont basic type, may be linked to an increase in the level of calcification of the carapace (Yamaguchi 2003).

Colonization and dispersal in continental groundwaters

The evolutionary origins of subterranean Recent ostracods are best understood as due to an interplay of vicariance and dispersal. Danielopol (1980) discussed in detail two main concurrent hypotheses to infer the antiquity of the genus *Pseudolimnocythere*. He applied the regression evolution model (Stock 1977) together with possible pathways of colonization of continental areas. Both hypotheses assumed that *Pseudolimnocythere* species colonized inland subterranean waters during marine regressions, ranging from the Upper Miocene – Lower Pliocene to Pleistocene. Afterward, Danielopol and Bonaduce (1990) suggested the Messinian salinity crisis as a key-event favouring *Pseudolimnocythere* colonization. Although at that time the known *Pseudolimnocythere* species had been found exclusively near the present coastline of the Mediterranean Sea, Danielopol (1980) hypothesized the possible discovery of *Pseudolimnocythere* species far away from coastlines due to their presumed ability of dispersal, mainly due to their small size. However, the subsequent discovery of Miocene (Danielopol et al. 1991) and Quaternary fossil and sub-fossil species (Bekkali and Nachite 2006; Aiello et al. 2020), and of further Recent species in continental groundwaters (Peterson et al. 2013; this paper) have brought useful elements to better hypothesize the time and mode of colonization routes of *Pseudolimnocythere*.

As reported by Savatzenalinton and Martens (2009), the hypothesis by Danielopol and Bonaduce (1990) of a main colonization event during the Messinian salinity crisis must be rejected, because there were already non-marine *Pseudolimnocythere* species in the Middle Miocene. Furthermore, Danielopol et al. (1991) contradicted the Messinian salinity crisis hypothesis for *P. hainburgensis*, a freshwater or brackish water species from the Badenian of the Vienna Basin, pointing out that this colonization must have taken place before. The Vienna Basin was subject to an important sea-level drop associated with an Antarctic cooling step 14.2 Ma (Rögl et al. 2007). More generally, the Middle Miocene of Europe was a time of paleogeographic reorganizations and strong tectonic activities, due to the orogeny and uplift of the Alpine-Himalayan chains and global climate change (Zachos et al. 2001). These changes also affected coastal marine environments, together with the intermittent opening and closing of marine waterways between Indian Ocean, Mediterranean, and Paratethys (Rögl 1999; Sant et al. 2017).

Moreover, *Pseudolimnocythere* sp. A Bekkali and Nachite 2003 from the Plio-Pleistocene basin of Saïss was associated with a true freshwater fauna. The presence of taxa such as *Candona angulata*, *Fabaeformiscandona fabaeformis*, *Potamocypris* sp. and *Darwinula* sp. indicated freshwaters rather rich in bicarbonate and slightly alkaline; these conditions are probably due to water coming from the Lias limestone and dolomite beds bordering the basin (Bekkali and Nachite 2006).

Recently, different biogeographical models have been explored to explain the distribution in the groundwaters of the Apennine and Balkanic peninsula, separated each other by the Adriatic Sea, of three different clades of stygobitic amphipods of the genus *Niphargus* Schiödt 1849. Among the considered scenarios, marine regression/transgression cycles resulted to be the most relevant events explaining their trans-Adriatic distribution, while transitional freshwater subterranean pathways created by landmass connections probably did not play an important role (Delić et al. 2020). The known distribution of the fossil and living species of *Pseudolimnocythere* seems to confirm this kind of vicariance model for this thalassoid genus, and the occurrence of repeated and independent events of colonization of continental waters linked to sea level variations since the Middle Miocene, can be hypothesized. Distributional data compared to Middle Miocene coastlines (Fig. 9) show a quite good correspondence between present distribution and the ancient coastlines, suggesting a previous dispersal stage along shorelines followed by multiple invasion routes rather than a single colonization event followed by dispersal. A further migration from subsurface interstitial to deeper groundwater habitats is of course possible.

The occurrence of repeated and independent colonizations and local speciation events seems to be further supported by the finding of *Pseudolimnocythere* species only in their type localities or, for *P. hypogaea*, in a small area, indicating that their dispersal ability is quite low. The only notable exception could be *P. hartmanni*, found in the Quaternary of southern Italy, and as extant species in a Greek Aegean Island, albeit this conspecificity can be questioned and needs to be confirmed.

Furthermore, it is worth noting that most *Pseudolimnocythere* species occur in correspondence with carbonate outcrops (Suppl. material 1: Fig. S5). Submarine karstic

springs in shallow marine areas have been proposed as convenient pathways for the colonization of inland subterranean habitats by marine lineages because their lower concentration of Na^+ ions typical of sea water is compensated by a higher concentration of Ca^{2+} ions (Danielopol and Bonaduce 1990; Notenboom 1991). This fact suggests that the karstic micro-crevices may be one of the main habitats exploited by *Pseudolimnocythere* species, as was clearly demonstrated in the study of Poiano karstic springs for *P. sofiae* sp. nov. (Stoch et al. 2009a).

Osmoregulatory mechanisms that make it possible to cross the salinity boundary (Aladin and Potts 1996) are of fundamental importance for the colonization of inland groundwater by marine ancestors. There are euryhaline Cytheroidea species living on the surface of shallow marine sediments which can be found in brackish and freshwater habitats too, like *Cyprideis torosa* Jones, 1950 (Meisch 2000). The ability to move upstream in small lotic environments is documented for some cytherid species, as *Tyrrhenocythere amnicola* (Sars, 1887) as shown by Pieri et al. (2015, 2020). Among living representatives of *Pseudolimnocythere*, possibly only *P. abdita* sp. nov. lives in freshwater, while the other extant species of this genus were found in habitats influenced by marine waters, with a salinity gradient from brackish to saline, or in sodic (as *P. sofiae* sp. nov.) and sulfidic (as *P.* sp. Peterson, 2013) waters. As reported above, also the fossil species *P. hainburgensis* and *P.* sp. A Bekkali & Nachite, 2003 were able to invade low-salinity environments.

Negative rheotaxis has been observed in different meiofaunal taxa, demonstrating movements directed upstream in the hyporheic zone (Bruno et al. 2012). For example, the presence of *P. abdita* sp. nov. at over 1000 m above sea level can be explained by the relative proximity of the crest of northern Apennine chain to the coastline during the late Pliocene, and by the presence of small watercourses which may have served as routes of colonization of groundwater ecosystems. Transport of stygobionts from inland subterranean habitats through surface or sub-surface outflow is another possible dispersal mechanism. A survey carried out at Poiano spring demonstrated that rapid discharge variation due to rainfall was responsible for the drift of up to 38 individuals per day of *Pseudolimnocythere sofiae* sp. nov. (Stoch et al. 2009a). However, the scattered records of individuals (mostly larvae) found downstream in the Secchia river, did not evidence the actual colonization of the interstitial habitats, indicating that the role of this mechanism may be of minor importance, even at a very small spatial scale.

Conclusions

Our analyses have been directed toward reassessing existing theories on evolution and biogeographic patterns of subterranean non-marine ostracods, based on the known distribution and new findings of species belonging to the genus *Pseudolimnocythere*. This allowed us to hypothesize different evolutive paths determined by geological events as well as by species morphological and ecological traits and their responses to selective pressures.

Limited accessibility to hypogean habitats determines a still fragmented knowledge of groundwater ostracod biodiversity. In addition, poor taxonomic resolution often hinders the possibility to identify patterns and scenarios at suitable space and time scales. Extensive research in hypogean environments will significantly increase our poor knowledge on ostracod diversity in groundwater and will contribute to a better understanding of their evolutionary and systematic relationships. Up to now, the rarity and difficulty to collect *Pseudolimnocythere* species prevented the application of DNA sequencing in studying this peculiar genus. There is no doubt that, along with a traditional morphological approach (indispensable when comparing fossil and Recent species), the use of molecular markers will disclose new possibilities for the investigation of the evolutionary history of subterranean ostracods.

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Supplementary material I

Figures S1–S5

Authors: Giampaolo Rossetti, Fabio Stoch, Ilaria Mazzini

Data type: Occurrences and images

Explanation note: Additional information on the sampled localities and the geographical distribution of the genus *Pseudolimnocythere*.

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The identities of two species in the *Pterostichus macrogenys* species group of subterranean carabid beetles (Coleoptera, Carabidae) revealed by external morphometric analysis and comparative genital morphology

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Abstract

The *Pterostichus macrogenys* species group is an endemic subterranean Japanese carabid lineage that shows marked regional differentiation, but unresolved taxonomic issues remain, even at the species level. Based on morphological examinations of the genital structures of newly collected specimens and an external morphometric analysis of all the species concerned, *P. falcispinus* Sasakawa, 2005 **syn. nov.** is synonymized with *P. asahinus* Habu & Baba, 1960, and *P. awashimaensis* **sp. nov.** is described from Awashima Island, a small island off the coast of Honshu. Based on external morphometrics, *P. awashimaensis* was determined to be most similar to *P. yahikosanus* Sasakawa, 2009, but its external and genital features differ distinctly from the latter species and it is thought to be more ancestral.

Keywords

Discriminant analysis, ground beetle, Japan, new species, new synonym, *Nialoe*

Introduction

The *macrogenys* species group of *Pterostichus* subgenus *Nialoe* Tanaka, 1958 (s. lat., i.e., *Nialoe* sensu Sasakawa 2021) is a Japanese endemic subterranean lineage in the beetle family Carabidae. This group shows marked differentiation in mountainous areas of Honshu, and ca. 30 species are currently recognized (Sasakawa et al. 2020). Members of the species group are flightless due to their atrophied hind wings, and are characterized by a large head with long mandibles and flattened body, which is thought to be associated with a subterranean lifestyle. Sasakawa et al. (2020) were the last to revise the group and revealed that coexisting different-sized species and disjunct distributions are more common than previously recognized (Fig. 1). However, some issues remained unresolved.

This study sought to resolve two taxonomic issues in the *macrogenys* species group: the relationship between *P. asahinus* Habu & Baba, 1960 and *P. falcispinus* Sasakawa, 2005, and the identity of the population on Awashima Island, a small island off the coast of Honshu. The fact that the holotype of *P. asahinus* and the available specimen from the Awashima population are both females, which provide less taxonomic information than males, has hampered resolution of these taxonomic issues. To overcome this, we performed external morphometric analysis, a useful taxonomic method that can also be applied to females (e.g., Sasakawa and Kubota 2007; Ober, and Connolly 2015; Ortuño et al. 2021), in addition to the usual genital morphology examinations.

Materials and methods

Specimens newly examined here were collected using subterranean baited traps, which were also used in our previous studies (Sasakawa and Itô 2017). This study applied discriminant analysis using external morphometrics of female specimens, morphological examinations of the endophallus of male genitalia of a male specimen newly collected near the type locality of *P. asahinus*, and morphological examinations of genital membranous parts of the female specimen from Awashima Island.

The discriminant analysis was used to investigate morphometrically whether the identity of the *P. asahinus* holotype and our newly defined *P. asahinus* is supported and to which species the Awashima specimen is most similar. The following specimens were examined together with the Awashima specimen and *P. asahinus* holotype: *P. adatarasanus* 3♀, *P. asahinus* (redefined here to include *P. falcispinus*) 17♀, *P. chokaisanus* 2♀, *P. eboshiyamanus* 3♀, *P. iwakiensis* 7♀, *P. monolineatus* 1♀, *P. ohsawacavus* 1♀, *P. takadateyamanus* 25♀, and *P. yabikosanus* 6♀. These samples include all species to which the Awashima specimen and *P. asahinus* holotype may belong and for which female specimens are available. Scaled photos obtained previously (Sasakawa et al. 2020), from the specimens newly examined here, and of the *P. asahinus* holotype in the type-specimen database of the National Agriculture and Food Research Organization (Division of Informatics and Inventory, Insect Systematics Unit, Institute for Agro-

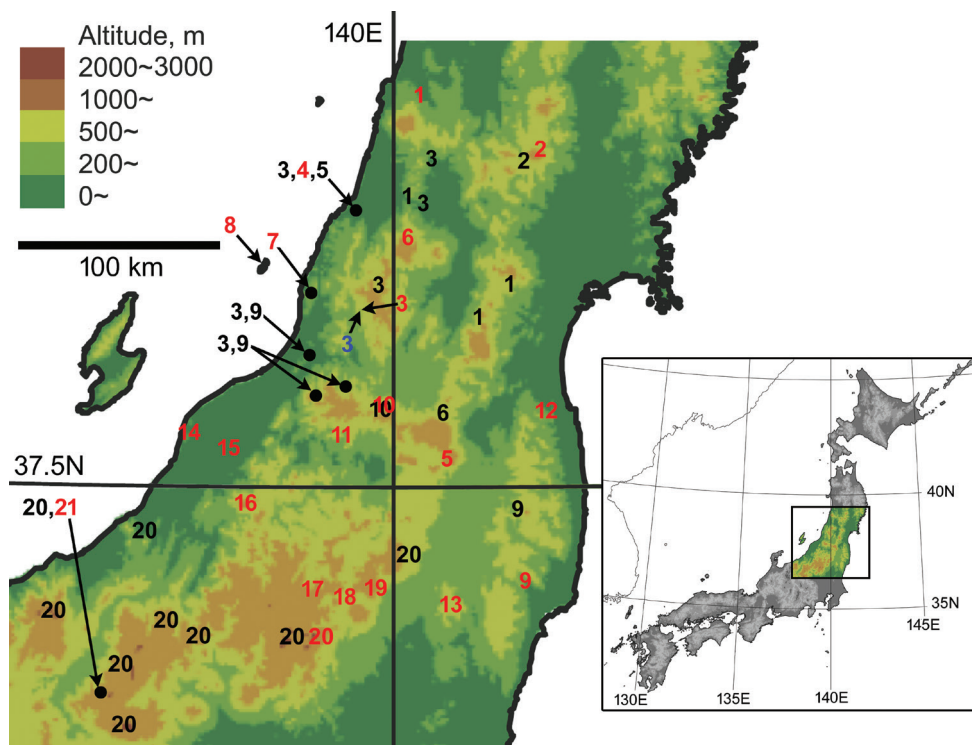


Figure 1. Distribution of the *Pterostichus macrogenys* species group in northern Chubu, northern Kanto, and southern Tohoku Districts, Honshu, based on specimens with unambiguous identities, modified from Sasakawa et al. (2020) and Satô (2021) **1** *P. chokaisanus* Sasakawa, 2009 **2** *P. kurikomasanus* Sasakawa, 2005 **3** *P. asahinus* Habu and Baba, 1960 **4** *P. takadateyamanus* Sasakawa, 2009 **5** *P. adatarasanus* Sasakawa, 2005 **6** *P. gassanus* Sasakawa, 2009 **7** *P. shinbodakensis* Sasakawa & Itô, 2017 **8** *P. awashimaensis* sp. nov. **9** *P. iwakiensis* Sasakawa, 2009 **10** *P. eboshiyamanus* Sasakawa, 2009 **11** *P. tateishiyamanus* Sasakawa & Itô, 2017 **12** *P. monolineatus* Sasakawa, Mitsuduka, & Itô, 2020 **13** *P. yamizosanus* Sasakawa, 2005 **14** *P. yabikosanus* Sasakawa, 2009 **15** *P. ohsawacavus* Sasakawa, 2005 **16** *P. sumondakensis* Sasakawa, 2005 **17** *P. isolatus* Sasakawa, 2005 **18** *P. nakamiyorinus* Morita, Ohkawa & Kurihara, 2013 **19** *P. momuranus* Morita, Ohkawa & Kurihara, 2013 **20** *P. macrogenys* Bates, 1883 **21** *P. nagasawai* Ito & Ogai, 2015. Red letters denote the type localities of each species. The blue letter indicates where the male specimen of *P. asahinus* newly examined here was collected.

Environmental Sciences, National Agriculture and Food Research Organization 2011) that were size-calibrated using information on the body length in the original description (15.2 mm; Habu and Baba 1960) were analyzed. Using these photos, four parts of the pronotum were measured with ImageJ v.1.50i (Rasband 2016) to the nearest 0.01 mm: pronotum length along the median line (PL), pronotal anterior margin width (PAW), pronotum width at the widest part (PW), and pronotal posterior margin width (PPW). Next, linear discriminant analysis was performed for a dataset excluding the Awashima

specimen and *P. asahinus* holotype, with “species” as the response variable and the four measurements as explanatory variables. Then, the species identities of the Awashima specimen and *P. asahinus* holotype were determined using the obtained function. To capture the results visually, scatterplots based on the first two canonical variates were created. All statistical analyses were performed in R v.3.4.3 (R Development Core Team 2017). Information on specimens and the raw data are in Appendix 1.

The male endophallus was everted and fully inflated by injecting toothpaste from the base of the aedeagus (Berlov 1992). For the female genitalia, muscles around the genitalia were dissolved using 5% potassium hydroxide, and the organs were cleaned and observed in pure water. The terminology of the male genitalia followed Sasakawa et al. (2020) and Sasakawa and Itô (2015). Both male and female genitalia were compared with photos of the organs of related species, which were taken during previous studies (Sasakawa and Itô 2015, 2017; Sasakawa et al. 2020).

Taxonomy

Pterostichus (Nialoe) asahinus Habu & Baba, 1960

Fig. 2

Pterostichus (Paralianoe) macrogenys asahinus: Habu and Baba (1960): 62 (original description), holotype ♀: “Mt. Dorokujin, Mts. Asahi, Niigata Pref.” [Miomote, Mt. Dôrokujinpô, Murakami-shi, Niigata Prefecture, Japan]; Habu and Baba (1972): 19.

Pterostichus (Paralianoe) asahinus: Habu (1977): 14 (part?).

Pterostichus macrogenys: Tanaka (1985): 114 (part?).

Pterostichus (Nialoe) asahinus: Bousquet (2017): 724.

Pterostichus (Nialoe) falcispinus: Sasakawa (2005): 75 (original description), holotype ♂: “Cave Ishikiri, Nakajo-Machi, N-Echigo” [Ishikiri Cave, Mt. Ishikiriyama, Haguro, Tainai-shi, Niigata Prefecture, Japan]; Bousquet (2017): 724; Sasakawa et al. (2020): 7. Syn. nov.

Specimen examined. 1♂, Miomote, alt. 276 m, on the right bank of Miomotegawa River, Murakami-shi, Niigata Prefecture, Japan (38.273211°N, 139.779922°E), 12.vi.–17.vii.2021, Hirotarô Itô leg., in the collection of HI.

Notes. Sasakawa et al. (2020) suggested that *P. asahinus* and *P. falcispinus* might be conspecific. However, this hypothesis remained untested because males from the type locality of *P. asahinus* have not been examined. The male specimen examined here was obtained from a valley southwest of Mt. Dôrokujinpô, which can virtually be regarded as the type locality (Fig. 1). The structures of the endophallus and right paramere of this specimen are identical to those of the eastern type of *P. falcispinus* in Sasakawa et al. (2020). Its body length is intermediate between that of the eastern and western types of *P. falcispinus* as follows: body length from mandible apices to elytral end (BLm) 16.32 mm, that from anterior margin of labrum (BLl) 14.72 mm, and

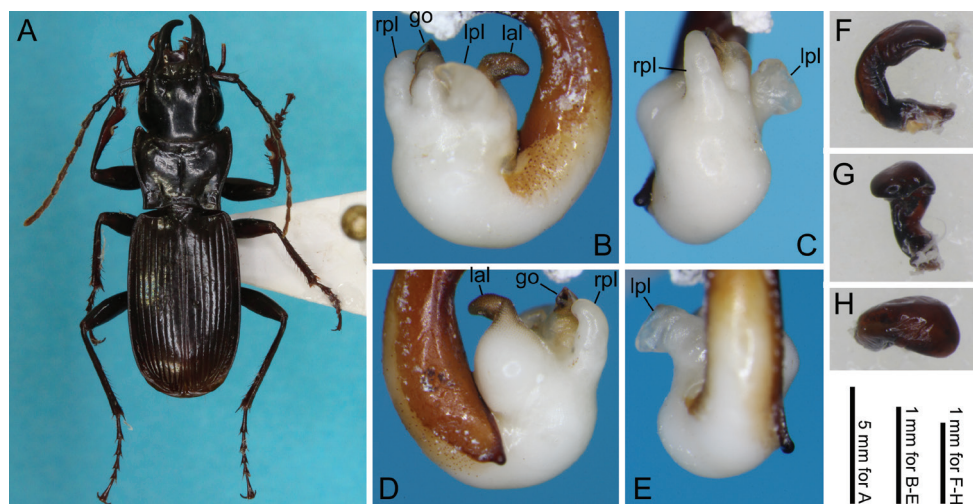


Figure 2. *Pterostichus asahinus* male from Miomote, on the right bank of the Miomotegawa River **A** habitus dorsal view **B–E** Endophallus left lateral (**B**) ventral (**C**) right lateral (**D**) and dorsal (**E**) views **F–H** right paramere left lateral (**F**) apical (**G**) and dorsal (**H**) views. **go**, gonopore; **lal**, left apical lobe; **lpl**, left preapical lobe; **rpl**, right preapical lobe.

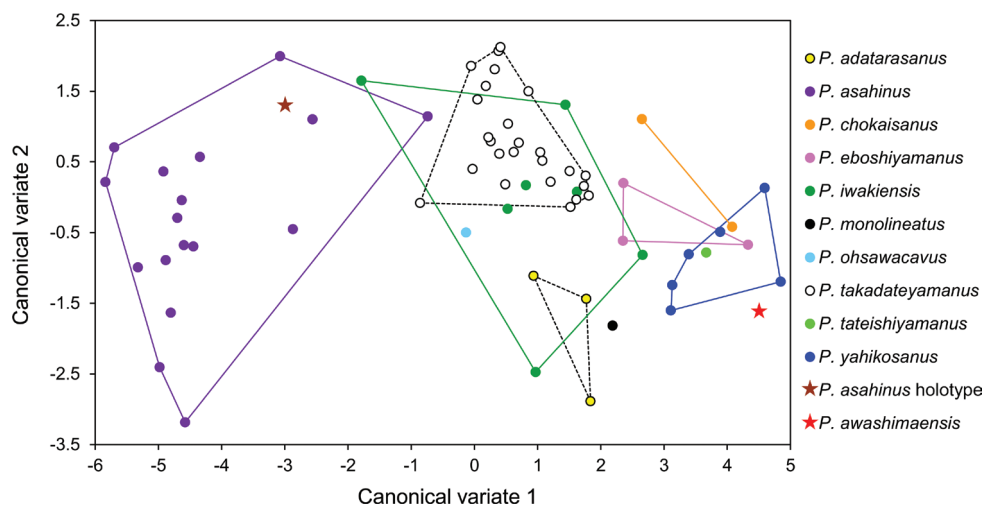


Figure 3. Scatterplot of the first two canonical variates obtained from the discriminant analysis of four measurements of the female pronotum.

that from clypeal apex (BLc) 14.26 mm. Based on these results, we regard *P. asahinus* and *P. falcispinus* as conspecific. The results of the discriminant analysis support this conclusion. The *P. asahinus* holotype was classified in this newly defined *P. asahinus* in the discriminant function analysis, and was within the area of *P. asahinus* on the scatterplot of the first two canonical variates (Fig. 3).

***Pterostichus (Nialoe) awashimaensis* Sasakawa & Itô, sp. nov.**

<http://zoobank.org/77C1F12B-3E95-4058-92B8-AA57D4F76D02>

Fig. 4

Pterostichus (Paralianoe) macrogenys macrogenys: Habu and Baba (1972): 19 (part).

Pterostichus macrogenys: Tanaka (1985): 114 (part?); Shimizu (2001): 23.

Type specimen. Holotype: ♀, Mt. Koshibayama, alt. 235 m, Awashima Island, Awashima-mura, Niigata Prefecture, Japan, 24.v.–14.vi.2015, Hirotarô Itô leg., deposited in the Laboratory of Zoology, Department of Science Education, Faculty of Education, Chiba University, Chiba, Japan.

Notes. In the discriminant function analysis, the Awashima specimen was classified as *P. yahikosanus*. On the scatterplots of the first two canonical variates, the specimen was located outside of, but close to, the area of *P. yahikosanus* (Fig. 3). These results suggest that the Awashima specimen is most similar in external morphology to, but different from, *P. yahikosanus*. Differences between the Awashima specimen and *P. yahikosanus* were also observed in other morphological features: the body length of the Awashima specimen (see below) is smaller than that of *P. yahikosanus* (n=6 from Mt. Yahikosan; BLm 15.32–14.35, mean 14.94 mm; BLl 14.22–13.46, mean 13.73 mm; BLc 13.64–12.89, mean 13.18 mm) (Fig. 4A, B); in the pronotum, anterior angles are barely produced, hind angles are obtuse, and posterior margin is arcuate posteriorly behind the laterobasal impressions in the Awashima specimen (Fig. 4C), whereas in *P. yahikosanus*, anterior angles are notably produced, hind angles are right-angled to acute, and the posterior margin behind the laterobasal impressions is almost straight (Fig. 4D); the body of the Awashima specimen is darker than that of *P. yahikosanus* (Fig. 4A–D); and pigmentation on the innermost part of vagina is smaller in the Awashima specimen, less than half the size of median and seminal apophyses (Fig. 4E), while it is larger than these apophyses in *P. yahikosanus* (Fig. 4F). In the *macrogenys* species group, pronotum shape and the pigmentation on the innermost part of vagina differ distinctly among species and are used as important species-level diagnostic characters (e.g., Sasakawa 2005; Sugimura 2005; Morita et al. 2013). Because these characters differ distinctly between the Awashima specimen and *P. yahikosanus*, we describe the Awashima specimen as a new species. Based on information on the morphological features of the pronotum and female genitalia in related species (Sasakawa et al. 2020 and references therein), notably produced pronotal anterior angles and developed vaginal pigmentation are limited to some species and considered to be derived character states in the *macrogenys* species group. Therefore, the Awashima species is considered more ancestral than *P. yahikosanus* with respect to the pronotal and genital features.

Description. BLm 13.79 mm; BLl 12.66 mm; BLc 12.13 mm. Head, pronotum, and elytra dark brown; appendages reddish brown. Dorsal surface almost smooth except for laterobasal impressions of the pronotum, the anterior half of which bear several transverse wrinkles and the posterior half of which are weakly punctate.

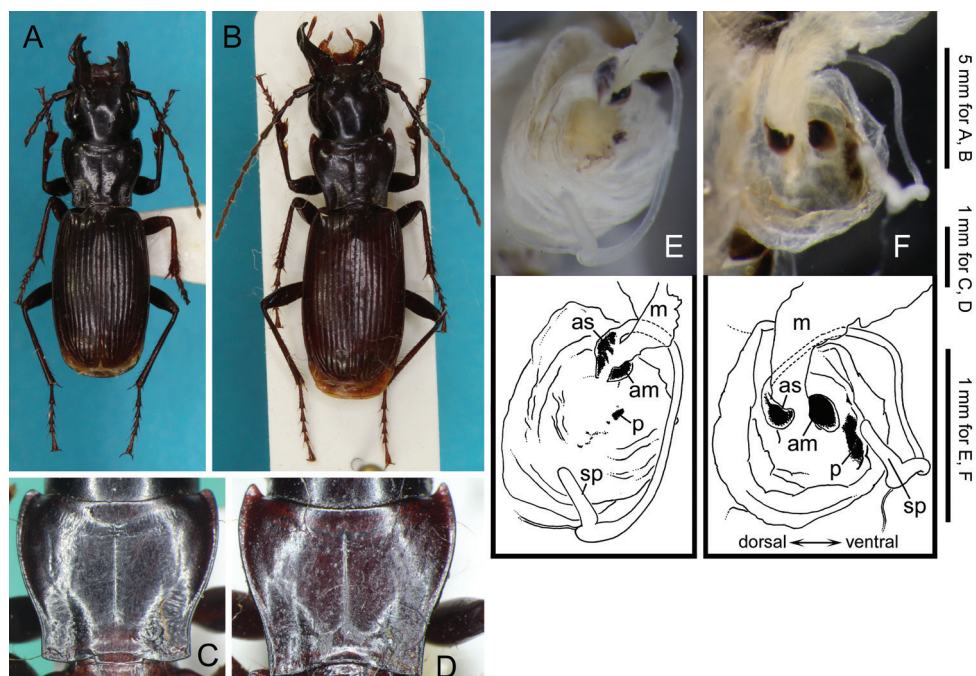


Figure 4. *Pterostichus awashimaensis* sp. nov. holotype female (**A, C, E**) and *P. yahikosanus* female from Mt. Yahikosan (**B, D, F**) **A, B** habitus dorsal view **C, D** pronotum dorsal view **E, F** vagina anterior view. **am**, apophysis of the median oviduct; **as**, apophysis of the seminal canal; **m**, median oviduct; **p**, pigmentation on the innermost part of the vagina; **sp**, spermatheca.

Head large, widest at tempora, which are distinctly swollen; width at the widest point larger than pronotal posterior margin width; length from clypeal apex to neck base longer than pronotum length along the median line. Left mandible larger than the right and curved at the apical 1/4; length between mandible apex and posterolateral end on dorsal side slightly shorter than 2.5 times the anterior width of the clypeus. Eyes weakly convex, with the anterior-posterior length longer than 1/2 length of antennal segment 1. Antennal segment 2 with two setae.

Pronotum cordate, notably flat, widest at apical 1/5. Lateral margins arcuate on apical 2/3, slightly sinuate on basal 1/3; two marginal setae on each lateral side, anterior setae near widest pronotal point, and posterior setae near hind angles. Anterior margin emarginated, with curvature approximately the same as that of apical 2/3 of lateral margins; anterior angles widely rounded and barely produced. Hind angles obtuse. Posterior margin only slightly emarginated at median area, weakly but distinctly arcuate posteriorly behind the laterobasal impressions. Median line impressed in the middle, not reaching either the anterior or posterior margins; laterobasal impressions single, shallow.

Elytra sides almost parallel, less convex; shoulder distinct, but not denticulate; apices rounded; scutellar stria present, connected to stria 1 on the right side but not on

the left; 1 setigerous puncture on stria 1 at the level of the posterior end of scutellum; two setigerous punctures on interval 3, anterior one slightly before the middle and posterior one on apical 1/5, both adjoining stria 2. Hind wings completely atrophied. First fore tarsomere no adhesive hairs on ventral side.

Female genital structures identical to those of other consubgenera; apophyses of seminal canal and median oviduct fully sclerotized; pigmentation on the innermost part of the vagina present, but less than half the size of apophyses of seminal canal and median oviduct; other parts of vagina lack conspicuous pigmentation.

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Appendix I

Table A1. Information on specimens examined and the raw data for discriminant analysis.

ID [†]	Species	Collection site [§]	PL	PAW	PW	PPW
mttk071	<i>adatarasanus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	2.82	3.55	3.93	3.08
mttk073	<i>adatarasanus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	2.85	3.64	4.16	2.97
mttk077	<i>adatarasanus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	2.66	3.40	3.91	2.93
cHIT_0004	<i>asahinus</i>	Ishikiri Cave, Tainai-shi, NI	2.83	3.98	4.48	3.44
mttk028	<i>asahinus</i>	Kurosawagawa forest road, Mamurogawa-machi, YG	2.43	3.42	3.94	3.07
mttk079	<i>asahinus</i>	Togawa, Tozawa-mura, YG	2.45	3.42	3.87	2.92
mttk026	<i>asahinus</i>	Ôtori, Tsuruoka-shi, YG	2.60	3.88	4.35	3.23
mttk040	<i>asahinus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	2.9	4.20	4.72	3.67
mttk070	<i>asahinus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	3.08	4.25	4.83	3.87
mttk072	<i>asahinus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	3.35	4.56	5.20	4.00
mttk033	<i>asahinus</i>	Nukumidaira, Oguni-machi, YG	3.14	4.66	5.08	3.98
mttk055	<i>asahinus</i>	Nukumidaira, Oguni-machi, YG	3.08	4.73	5.13	3.96
mttk056	<i>asahinus</i>	Nukumidaira, Oguni-machi, YG	2.98	4.37	4.88	3.71
mttk057	<i>asahinus</i>	Nukumidaira, Oguni-machi, YG	3.10	4.46	4.92	3.90
mttk059	<i>asahinus</i>	Nukumidaira, Oguni-machi, YG	2.97	4.35	4.77	3.76
mttk060	<i>asahinus</i>	Nukumidaira, Oguni-machi, YG	2.82	4.16	4.65	3.56
mttk061	<i>asahinus</i>	Nukumidaira, Oguni-machi, YG	3.18	4.39	5.19	3.74
mttk062	<i>asahinus</i>	Nukumidaira, Oguni-machi, YG	3.23	5.06	5.43	4.04
mttk063	<i>asahinus</i>	Nukumidaira, Oguni-machi, YG	3.08	4.37	5.00	3.73
mttk064	<i>asahinus</i>	Nukumidaira, Oguni-machi, YG	3.05	4.67	5.04	3.85
mttk030	<i>chokaisanus</i>	Sakunami, Sendai-shi, MG	2.54	3.07	3.61	2.46
mttk080	<i>chokaisanus</i>	Mt. Chokaisan, Yuza-machi, YG	2.25	2.86	3.34	2.40
mttk052	<i>eboshiyamanus</i>	Yunosawa, Iide-machi, YG	2.47	3.02	3.43	2.47
mttk053	<i>eboshiyamanus</i>	Yunosawa, Iide-machi, YG	2.38	3.09	3.47	2.61
mttk054	<i>eboshiyamanus</i>	Yunosawa, Iide-machi, YG	2.63	3.47	3.81	2.83
cHIT_0002	<i>iwakensis</i>	Miyahisa, Tainai-shi, NI	2.53	3.37	3.82	2.84
cHIT_0005	<i>iwakensis</i>	Ishikiri Cave, Tainai-shi, NI	2.38	3.35	3.87	2.93
iwakensisPT [†]	<i>iwakensis</i>	Iritôno, Iwaki-shi, FS	2.95	3.60	4.29	3.07
mttk034	<i>iwakensis</i>	Nukumidaira, Oguni-machi, YG	2.43	3.04	3.65	2.73
mttk058	<i>iwakensis</i>	Nukumidaira, Oguni-machi, YG	2.31	3.06	3.53	2.57
mttk065	<i>iwakensis</i>	Nukumidaira, Oguni-machi, YG	2.60	3.36	3.90	2.75
mttk066	<i>iwakensis</i>	Nukumidaira, Oguni-machi, YG	2.61	3.32	3.75	2.74
mttk037	<i>monolineatus</i>	Aobaminami, Marumori-machi, MG	2.77	3.55	3.94	2.96
cHIT_0053	<i>ohsawacavus</i>	Ohsawa Cave, Gosen-shi, NI	2.79	3.98	4.22	3.26
cHIT_0017	<i>takadateyamanus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	2.37	3.06	3.53	2.64
cHIT_0018	<i>takadateyamanus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	2.52	3.39	3.84	2.79
cHIT_0019	<i>takadateyamanus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	2.60	3.57	4.01	2.88
cHIT_0020	<i>takadateyamanus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	2.59	3.57	3.99	2.98
cHIT_0021	<i>takadateyamanus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	2.56	3.57	3.96	2.93
cHIT_0022	<i>takadateyamanus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	2.60	3.48	3.86	2.86
cHIT_0023	<i>takadateyamanus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	2.34	3.09	3.51	2.69
cHIT_0024	<i>takadateyamanus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	2.80	3.93	4.19	3.02
cHIT_0025	<i>takadateyamanus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	2.63	3.53	4.07	3.05
cHIT_0026	<i>takadateyamanus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	2.31	3.20	3.64	2.69
cHIT_0027	<i>takadateyamanus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	2.60	3.57	4.01	2.92
cHIT_0028	<i>takadateyamanus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	2.50	3.44	3.87	2.87
cHIT_0029	<i>takadateyamanus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	2.46	3.42	3.87	2.83
cHIT_0030	<i>takadateyamanus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	2.40	3.24	3.65	2.78
cHIT_0031	<i>takadateyamanus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	2.61	3.50	3.90	2.82

ID [†]	Species	Collection site [§]	PL	PAW	PW	PPW
cHIT_0032	<i>takadateyamanus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	2.52	3.70	4.01	2.94
cHIT_0034	<i>takadateyamanus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	2.72	3.73	4.10	2.90
cHIT_0035	<i>takadateyamanus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	2.46	3.44	3.83	2.77
cHIT_0036	<i>takadateyamanus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	2.51	3.32	3.81	2.84
cHIT_0037	<i>takadateyamanus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	2.25	3.07	3.52	2.67
cHIT_0039	<i>takadateyamanus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	2.46	3.31	3.67	2.80
mttk041	<i>takadateyamanus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	2.67	3.59	3.99	2.88
mttk074	<i>takadateyamanus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	2.59	3.76	4.14	2.89
mttk075	<i>takadateyamanus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	2.50	3.60	3.99	2.82
mttk076	<i>takadateyamanus</i>	Mt. Takadateyama, Tsuruoka-shi, YG	2.55	3.59	3.95	2.90
tateishiyamanusPTf [‡]	<i>tateishiyamanus</i>	Kuratani-sawa, Nishiaizu-machi, FS	2.50	3.07	3.51	2.55
cHIT_0044	<i>yahikosanus</i>	Mt. Yahikosan, Nagaoka-shi, NI	2.56	2.98	3.52	2.42
cHIT_0045	<i>yahikosanus</i>	Mt. Yahikosan, Nagaoka-shi, NI	2.59	3.18	3.65	2.67
cHIT_0046	<i>yahikosanus</i>	Mt. Yahikosan, Nagaoka-shi, NI	2.55	3.15	3.62	2.53
cHIT_0048	<i>yahikosanus</i>	Mt. Yahikosan, Nagaoka-shi, NI	2.50	3.10	3.56	2.40
cHIT_0049	<i>yahikosanus</i>	Mt. Yahikosan, Nagaoka-shi, NI	2.68	3.29	3.77	2.74
cHIT_0050	<i>yahikosanus</i>	Mt. Yahikosan, Nagaoka-shi, NI	2.62	3.29	3.73	2.66
asahinusHT [‡]	<i>asahinus</i>	Mt. Dōrokujinpō, Murakami-shi NI	2.51	3.51	4.14	3.10
awashima_F01 [‡]	<i>awashimaensis</i>	Awashima Island, Awashimaura-mura, NI	2.50	2.92	3.38	2.48

[†] cHIT_0002–39 and mttk026–77: specimens used in Sasakawa et al. (2020); cHIT_0044–53 in the collection of HI, mttk079–80 in the collection of Y. Mitsuduka (Yamagata), iwakiensisPT, tateishiyamanusPTf, and awashima_F01: specimens newly examined here.

[‡] Type specimens (paratype for *P. iwakiensis* and *P. tateishiyamanus*, and holotype for *P. asahinus* and *P. awashimaensis*).

[§] Prefecture abbreviations: FS, Fukushima Prefecture; MG, Miyagi Prefecture; NI, Niigata Prefecture; YG, Yamagata Prefecture.

A new subspecies of the genus *Duvalius* Delarouzée, 1859 (Coleoptera, Carabidae, Trechini) from western Serbia, with a key and an annotated catalogue of Serbian *Biharotrechus* and *Duvalius* s. str. taxa

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Abstract

Duvalius semecensis tarensis **ssp. nov.** from two subterranean sites situated on Mt. Tara (western Serbia) is described, illustrated and compared with its most related congeners. It is provisionally placed in the subgenus *Biharotrechus* Bokor, 1922. The new subspecies is characterized by a depigmented, medium-sized body, the presence of reduced eyes, deep and complete frontal furrows, two pairs of discal setae in third elytral striae, as well as by the shape of aedeagus. It inhabits caves on Mt. Tara and is endemic of this mountain. Data on the distribution and bionomy of the new subspecies are given. Its closest relative, *Duvalius* (*Biharotrechus*) *semecensis semecensis* Winkler, 1926, is redescribed and we designated its holotype by monotypy. A key for the identification and an annotated catalogue of Serbian *Biharotrechus* and *Duvalius* s. str. taxa are also provided.

Keywords

Ground beetles, new taxon, Serbian Dinarides, subterranean fauna, Trechinae

Introduction

The territory of western Serbia is inhabited by a number of mesovoid shallow substratum (MSS)-dwelling and subterranean trechine taxa (Belousov 2017; Hlaváč et al. 2017), but limited to the MSS and certain caves and pits located in the Dinaric mountain chain of Serbia (Ćurčić et al. 2016, 2018a). The hypogean trechine fauna of western Serbia has been studied for almost a century (Jeannel 1923, 1928), but intensive systematic explorations have been conducted in the last two decades, resulting in the descriptions of numerous taxa of the genus *Duvalius* Delarouzée, 1859 new to science (Guéorguiev et al. 2000; Ćurčić et al. 2001, 2003a, 2003b, 2005, 2016; Pavićević and Popović 2001, 2003; Janák and Moravec 2008). Nevertheless, the karstic areas of Mt. Tara and its surroundings in western Serbia are insufficiently biospeleologically investigated so far. Recently, a new genus (*Velesaphaenops* Ćurčić & Pavićević, 2018) and a new species (*V. tarensis* Ćurčić & Pavićević, 2018) of aphaenopsoid trechine beetles were described from a pit on Mt. Tara (Ćurčić et al. 2018a). Another aphaenopsoid trechine beetle, *Acheroniotes lethensis* Ćurčić & Pavićević, 2018, inhabits subterranean habitats in Kamena Gora near Prijepolje (southwestern Serbia) (Ćurčić et al. 2018a).

Several field trips conducted by the third (MK) and the fourth author (FB) at two subterranean sites on Mt. Tara resulted in the discovery of a series of specimens belonging to the genus *Duvalius* Delarouzée, 1859. Subsequent analysis of the beetles yielded that they belong to a *Duvalius* subspecies unknown to science, which is described and diagnosed in this contribution.

Materials and methods

The diagnosis of *Duvalius* (*Biharotrechus*) *semecensis tarensis* ssp. nov. is based on the study of type series, which consists of 13 specimens (two males and 11 females). Specimens were collected by pitfall trapping during 2013, 2014 and 2017 at two subterranean sites on Mt. Tara (an unnamed cave in the village of Solotuša and the pit 4-1-3-27 in the village of Kaluđerske Bare) (Fig. 1). Pitfall traps were baited with rotten meat.

The specimens were studied by the first (SĆ) and the second author (NV) in the laboratory of the Institute of Zoology, University of Belgrade - Faculty of Biology, Belgrade, Serbia. Studied specimens were dissected, analyzed and illustrated. Dry beetles were glued onto separate paper mounting cards. Morphological structures of the beetles were examined using Nikon SMZ 800N and Zeiss Stemi 508 stereomicroscopes. Male and female genitalia were dissected, cleaned in KOH, fixed in a medium consisting of Canada balsam and xylol, mounted on transparent plastic mounting cards and pinned under examined specimens. Macro photographs were taken by a Nikon SMZ 800N stereomicroscope with a Nikon DS-Fi2 digital camera and improved using Adobe Photoshop CS6 software. A Nikon DS-L3 control unit was used for scaling.

The systematics used follow Jeannel (1928), Belousov (2017) and Hlaváč et al. (2017).

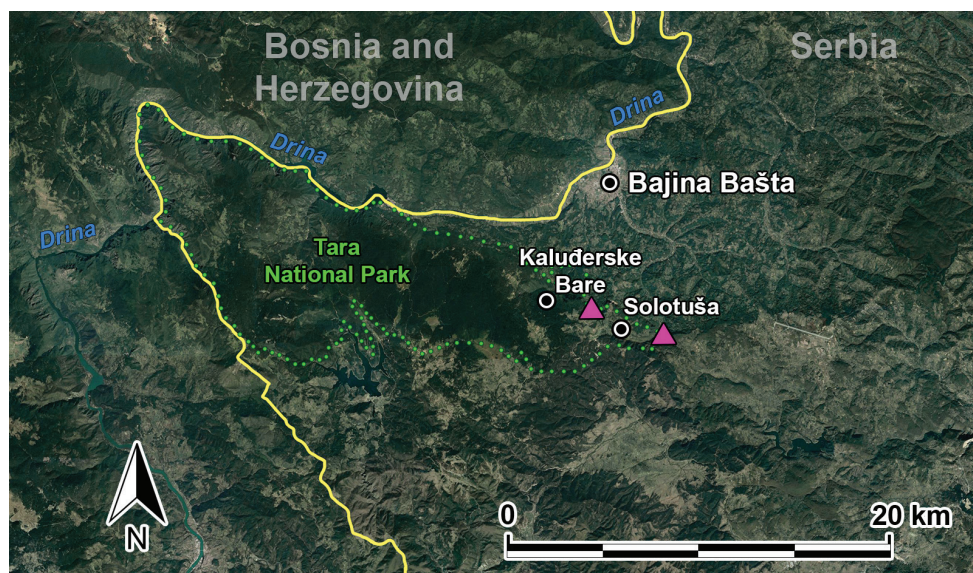


Figure 1. A map of the Tara National Park (western Serbia) with sites (pink triangles) where *Duvalius* (*Biharotrechus*) *semecensis tarensis* ssp. nov. was found.

Abbreviations of measurements

A2L/A2W	ratio of length to width of antennomere 2;
A10L/A10W	ratio of length to width of antennomere 10;
AEL	length of aedeagus;
EL	length of elytra (measured along suture from base to apex);
EL/EW	ratio of length of elytra to maximum width of elytra;
EW	maximum width of elytra;
HL	length of head (measured from the anterior margin of clypeus to neck constriction);
HL/HW	ratio of length of head to maximum width of head;
HW	maximum width of head;
L	overall body length (measured from the apex of mandibles to the apex of elytra along suture);
PL	length of pronotum (measured along median line);
PL/PW	ratio of length of pronotum to maximum width of pronotum as greatest transverse distance;
PW	maximum width of pronotum as greatest transverse distance;
TL	total body length (measured from the anterior margin of clypeus to the apex of elytra along suture).

Collections

CDP	private collection of Dragan Pavićević, Belgrade, Serbia;
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INCS	collection of the Institute for Nature Conservation of Serbia, Belgrade, Serbia;
IZFB	collection of the Institute of Zoology, University of Belgrade - Faculty of Biology, Belgrade, Serbia;
NHMV	collection of the Natural History Museum Vienna, Vienna, Austria.

Other abbreviations

AW	leg. Augusta Weirather;	IN	leg. Iva Njunjić;
BM	leg. Bojan Mitić;	MK	leg. Miloš Kuraica;
DP	leg. Dragan Pavićević;	MKO	leg. Marjan Komnenov;
DR	leg. Dejan Radović;	MP	leg. Momčilo Popović;
ĐM	leg. Đorđe Marković;	MSS	mesovoid shallow substratum;
FB	leg. Fabrizio Bosco;	PT	paratype;
GN	leg. Guido Nonveiller;	SC	leg. Srećko Ćurčić;
HT	holotype;	SO	leg. Siniša Ognjenović.
ibid.	in the same place;		

Other examined taxa

Duvalius (Biharotrechus) reufi Pavićević & Popović, 2003: one female, southwestern Serbia, town of Sjenica, Pešter Plateau, village of Ušak, Ušak Cave System, 30.VIII.2002, DP (CDP).

Duvalius (Biharotrechus) semecensis semecensis Winkler, 1926: HT male, eastern Bosnia and Herzegovina, town of Višegrad, Mt. Sjemeć, village of Ćipi, Vrteljka Cave, with no date provided, AW (NHMV).

Duvalius (Duvalius) bolei Pretner, 1963: two males, four females, southeastern Serbia, town of Svrlijig, Svrlijske Planine Mts., village of Prekonoga, Prekonoška Pećina Cave, 10.V–27.X.2004, pitfall traps, SO (CDP); one female, *ibid.*, 11.V.2004, DP (CDP); one male, *ibid.*, 27.X.2004, DP (CDP); one male, *ibid.*, 28.V.2013, ĐM (IZFB).

Duvalius (Duvalius) javorensis (S. Ćurčić, Brajković & B. Ćurčić, 2003): HT male and four PT females, southwestern Serbia, town of Nova Varoš, Mt. Javor, village of Trudovo, Pećina pod Kapilijama Cave, 20.VIII.2003, SC (IZFB); two PT females, *ibid.*, VIII.2002, SC & BM (IZFB).

Duvalius (Duvalius) sturanyi sturanyi (Apfelbeck, 1904): one male, two females, western Serbia, town of Ljubovija, Mt. Bobija, 1,000 m a.s.l., 26.V.1981, GN (CDP); one female, northern Montenegro, town of Žabljak, Mt. Durmitor, Tara River Canyon, Splavište, Pećina nad Splavištem Cave, 2.VII.1991, DP (CDP); three females, southwestern Serbia, town of Prijepolje, village of Babine, Velika Pećina Cave, 1,166 m a.s.l., 25.VII.2009, MKO & MP (CDP); one male, one female, *ibid.*, 6.VII.2010, IN & SO (CDP); two males, *ibid.*, 6.VII.2010–25.XI.2011, pitfall traps, SO & IN (CDP).

Duvalius (*Duvalius*) *suvoborensis* Pavićević & Popović, 2001: HT male and six PT females, western Serbia, town of Valjevo, Mt. Suvobor, Rajac, village of Brezaci, Pećina u Brezacima Cave, 5.VI–3.XII.2002, pitfall traps, DP (INCS, CDP); one male, western Serbia, town of Valjevo, Mt. Suvobor, Ravna Gora, Mokra Pećina Cave, 690 m a.s.l., 6.VI–3.XII.2002, pitfall traps, DP (CDP); one male, western Serbia, town of Valjevo, Mt. Suvobor, Rajac, Mala Bezdan Pit, 15.VIII.2003, pitfall traps, SO (CDP).
Duvalius (*Duvalius*) *suvodolensis* (S. Ćurčić, Brajković & B. Ćurčić, 2003): HT female, southwestern Serbia, town of Nova Varoš, Mt. Javor, village of Debelja, Suvodol valley, 2.V.2003, SC, BM & DR (IZFB).

Results

Order Coleoptera Linnaeus, 1758

Family Carabidae Latreille, 1802

Subfamily Trechinae Bonelli, 1810

Tribe Trechini Bonelli, 1810

Genus *Duvalius* Delarouzée, 1859

***Duvalius* (*Biharotrechus*) *semecensis* Winkler, 1926**

***Duvalius* (*Biharotrechus*) *semecensis tarensis* Ćurčić & Vesović, ssp. nov.**

<http://zoobank.org/C15F5E6E-BB95-4135-B6F3-CB01BA659DC4>

Figs 2–8

Type material. *Holotype*: male (IZFB) labeled as follows: „WESTERN SERBIA: town of Bajina Bašta, Mt. Tara, village of Solotuša, an unnamed cave, 43°53'46.023"N, 19°36'1.339"E, 20.V–5.XI.2017, pitfall traps, MK“ (white label, printed) / „*Holotypus Duvalius* (*Biharotrechus*) *semecensis tarensis* ssp. nov. Ćurčić & Vesović det. 2021“ (red label, printed) (Fig. 2).

Paratypes (12 specimens). The same data as for HT [seven females, IZFB]; the same data as for HT except for date and collector [two females, IZFB, September, 17, 2013, FB]; one male and one female (IZFB) labeled as follows: „WESTERN SERBIA: town of Bajina Bašta, Mt. Tara, village of Kaluđerske Bare, pit 4-1-3-27, 43°54'30.712"N, 19°33'11.585"E, 20.V–5.XI.2017, pitfall traps, MK“; the same data as for the two preceding PTs except for date and collector [one female, IZFB, July, 5, 2014, FB]. All PTs are labeled with white, printed locality labels and with red printed labels „*Paratypus Duvalius* (*Biharotrechus*) *semecensis tarensis* ssp. nov. Ćurčić & Vesović det. 2021“.

***Etymology*.** The new subspecies is named after its *terra typica* – Mt. Tara in western Serbia.

Diagnosis and taxonomical remarks. A medium-sized (TL 4.31–4.67 mm, L 4.83–5.18 mm), glabrous, depigmented trechine beetle with the morphological character states of the subgenus *Biharotrechus*, genus *Duvalius* (see the Discussion). Colour

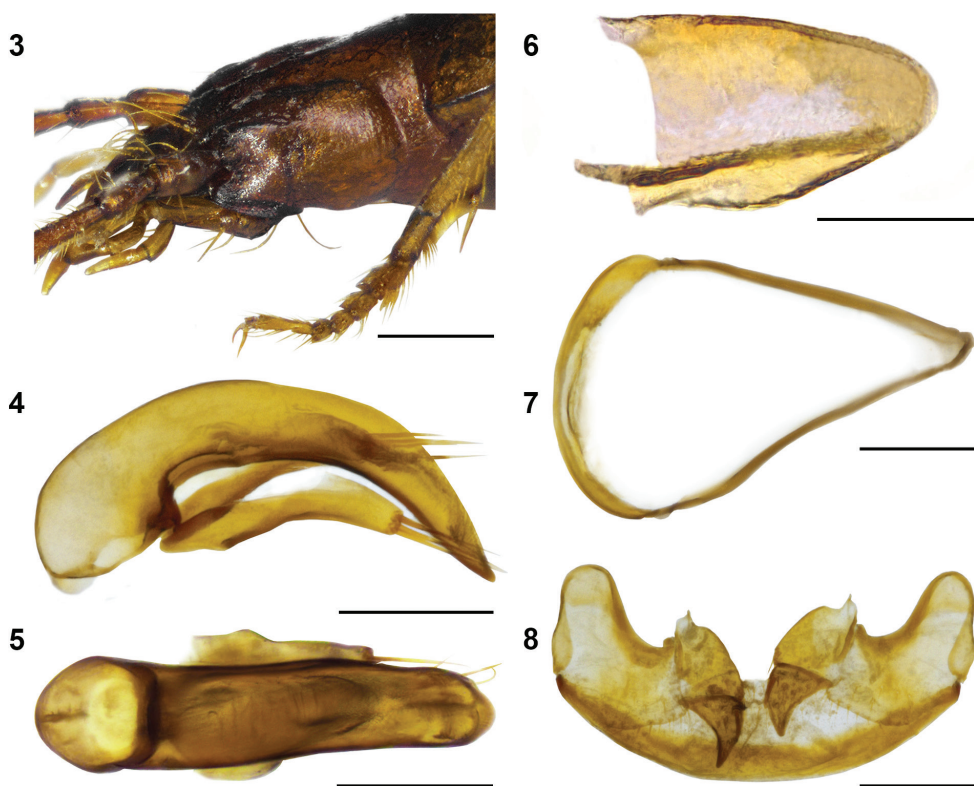


Figure 2. *Duvalius (Biharotrechus) semecensis tarensis* ssp. nov. from an unnamed cave, village of Solotuša, near the town of Bajina Bašta, Mt. Tara, western Serbia. HT male, habitus, dorsal aspect. Scale bar: 2 mm.

reddish-brown, head relatively large, rounded, with deep, complete frontal furrows and vestigial eyes. Pronotum transverse, heart-shaped, elytra sub-oval, with two pairs of discal setae (Figs 2, 3).

Duvalius (Biharotrechus) semecensis tarensis ssp. nov. is most closely related to the nominotypic subspecies of *Duvalius (Biharotrechus) semecensis* Winkler, 1926 from eastern Bosnia and Herzegovina (cave- and MSS-dwelling, from Mt. Sjemeć, near the town of Višegrad), *Duvalius (Biharotrechus) reufi* Pavićević & Popović, 2003 from southwestern Serbia (cave-dwelling, from the Pešter Plateau and Mt. Javor, near the towns of Sjenica and Nova Varoš), as well to the following three *Duvalius* species distributed in western and southwestern Serbia: *Duvalius (Duvalius) suvoborensis* Pavićević & Popović, 2001 (cave-dwelling, from Mt. Suvobor, near the town of Valjevo), *D. (D.) javorensis* (S. Ćurčić, Brajković & B. Ćurčić, 2003) (cave-dwelling, from Mt. Javor, near the town of Nova Varoš) and *D. (D.) suvodolensis* (S. Ćurčić, Brajković & B. Ćurčić, 2003) (MSS-dwelling, from Mt. Javor, near the town of Nova Varoš) (Figs 9, 10, 14, 16, 18, 19) (Winkler 1926; Jeannel 1928; Pavićević and Popović 2001, 2003; Ćurčić et al. 2003a, 2003b). All the listed taxa share the following morpho-anatomical features: they possess vestigial eyes, sharp hind pronotal angles and a specific unified gutter-formed copulatory piece. In *D. (B.) semecensis tarensis* ssp. nov. elytral base is somewhat oblique and shoulders are sloped and rounded, in *D. (B.) semecensis semecensis* Winkler, 1926 elytral base is strongly oblique and shoulders are sloped and obtuse, while in the remaining four species elytral base is almost straight and shoulders are elevated and rounded. Other differences between the new subspecies and its most related congeners are listed below.

From the HT of *D. (B.) semecensis semecensis* it is differed by the TL (4.17 mm vs. 4.31–4.67 mm), length and ratio of length to width of certain antennomeres (antennomere 11 longest, followed by antennomeres 1 and 3, A2L/A2W 1.89, A10L/A10W 2.60 vs. antennomere 3 longest, followed by antennomere 11, A2L/A2W 2.40, A10L/A10W 2.67–2.72), shape of eyes (oval vs. lenticular), number of ommatidia (1–2 vs. 5–8), position of maximum width of pronotum (slightly before anterior third vs. at anterior fourth), shape of lateral pronotal margins before hind pronotal angles (more sinuated vs. less sinuated), shape of lateral pronotal furrows (relatively narrow and shallow vs. wide and deep), position of anterolateral pair of pronotal setae (slightly before anterior third of pronotal length vs. in anterior fifth of pronotal length), shape and ratio of length to width of elytra (more elongate, sub-ovate, EL/EW 1.65 vs. less elongate, sub-oval, EL/EW 1.51–1.60), position of maximum width of elytra (slightly after middle vs. at middle), position of the first pair of elytral discal setae (slightly before level of third humeral seta vs. slightly below level of second humeral seta), position of the second pair of elytral discal setae (after middle vs. at middle or slightly above), shape of median lobe (thinner, more curved, apex more acute in lateral view vs. wider, less curved, apex less acute in lateral view) and basal bulb of aedeagus (moderately elongate, somewhat narrowed distally in lateral view vs. large, rounded) and shape of copulatory piece (edges largely folded dorsally to form a deep cone, without any projection vs. in form of a shallow gutter,



Figures 3–8. *Duvalius (Biharotrechus) semecensis tarensis* ssp. nov. from Mt. Tara, near the town of Bajina Bašta, western Serbia **3** HT male, head, lateral aspect **4** HT male, aedeagus, lateral aspect **5** HT male, aedeagus, dorsal aspect **6** HT male, copulatory piece, dorsal aspect **7** HT male, abdominal sternite IX (urite) **8** PT female, genitalia, dorsal aspect. Scale bars: 0.5 mm (**3**); 0.2 mm (**4, 5, 7, 8**); 0.1 mm (**6**).

with a longitudinal sclerotized projection, edges not folded and sclerotized) (Table 1, Figs 10, 11) (Winkler 1926; Jeannel 1928).

From *D. (B.) reufi*, *D. (B.) semecensis tarensis* ssp. nov. is differed by the body shape (more elongate vs. less elongate), HL/HW (head somewhat longer than wide vs. head wider than long), number of ommatidia (none vs. 5–8), width of genae (wider vs. narrower), antennal length (extending the middle of elytra or longer vs. not reaching the middle of elytra), shape of pronotum (more transverse vs. less transverse), position of maximum width of pronotum (at anterior third vs. at anterior fourth), shape of elytra (sub-parallel vs. sub-oval), shape of the median lobe (more curved, straight in distal half, with rounded apex in lateral view and narrowing distally in dorsal view vs. less curved, curved in distal half, with pointed apex in lateral view and sub-parallel in dorsal view) and basal bulb of aedeagus (elongate, narrowed distally in lateral view vs. large, rounded) and shape of copulatory piece (elongate, narrowed distally, without any projection, deeply incised proximally vs. wide, rounded distally, with a longitudinal sclerotized projection, shallowly incised proximally) (Fig. 14) (Pavićević and Popović 2003).

Table 1. Linear measurements and morphometric ratios of *Duvalius (Biharotrechus) semecensis tarensis* ssp. nov. and its closest conspecific relative *D. (B.) semecensis semecensis*.

Character	Subspecies	
	<i>Duvalius (Biharotrechus) semecensis tarensis</i>	<i>Duvalius (Biharotrechus) semecensis semecensis</i>
	ssp. nov.	
TL*	4.31–4.67	4.17
HL*	0.70–0.75	0.67
HW*	0.80–0.87	0.79
HL/HW	0.85–0.88	0.85
A2L/A2W	2.40	1.89
A10L/A10W	2.67–2.72	2.60
PL*	0.84–0.90	0.83
PW*	0.99–1.07	1.00
PL/PW	0.84–0.85	0.83
EL*	2.46–2.87	2.56
EW*	1.63–1.80	1.55
EL/EW	1.51–1.60	1.65

* – values in mm.

From *D. (D.) suvoborensis*, *D. (B.) semecensis tarensis* ssp. nov. is differed by the body shape (more elongate vs. less elongate), HL/HW (head somewhat longer than wide vs. head wider than long), number of ommatidia (about 10 vs. 5–8), antennal length (extending the middle of elytra or longer vs. not reaching the middle of elytra), shape of lateral pronotal margins at largest pronotal width (obtusely rounded vs. regularly rounded), shape of hind pronotal angles (less sharp vs. sharper), shape of the median lobe of aedeagus (apex less distally sub-apically elevated in lateral view vs. apex more distally sub-apically elevated in lateral view) and shape of copulatory piece (sub-parallel, with an obtuse apex, a deeper gutter and without any projection, not incised proximally vs. apically narrowed, with a rounded apex, a shallower gutter and a longitudinal sclerotized projection, shallowly incised proximally) (Fig. 18) (Pavićević and Popović 2001).

From *D. (D.) javorensis*, the new subspecies is differed by the head size in relation to the body (more voluminous vs. less voluminous), width of genae (wider vs. narrower), antennal length (extending over the middle of elytra vs. not reaching the middle of elytra), number of ommatidia (10–14 vs. 5–8), position of maximum width of pronotum (at anterior third vs. at anterior fourth) and elytra (below middle vs. at middle), shape of the median lobe of aedeagus (more robust, not narrowing apically, apex obtuse in dorsal view vs. less robust, narrowing apically, apex rounded in dorsal view) and shape of copulatory piece (sub-parallel, with an obtuse apex, a deeper gutter and without any projection vs. apically narrowed, with a rounded apex, a shallower gutter and a longitudinal sclerotized projection) (Fig. 16) (Ćurčić et al. 2003b).

From *D. (D.) suvodolensis*, *D. (B.) semecensis tarensis* ssp. nov. is differed by the HL/HW (head somewhat longer than wide vs. head wider than long), number of ommatidia (about 17 vs. 5–8), shape of lateral elytral margins (arcuate, sub-parallel vs. rounded) and position of maximum width of elytra (below middle vs. at middle) (Fig. 19) (Ćurčić et al. 2003a).

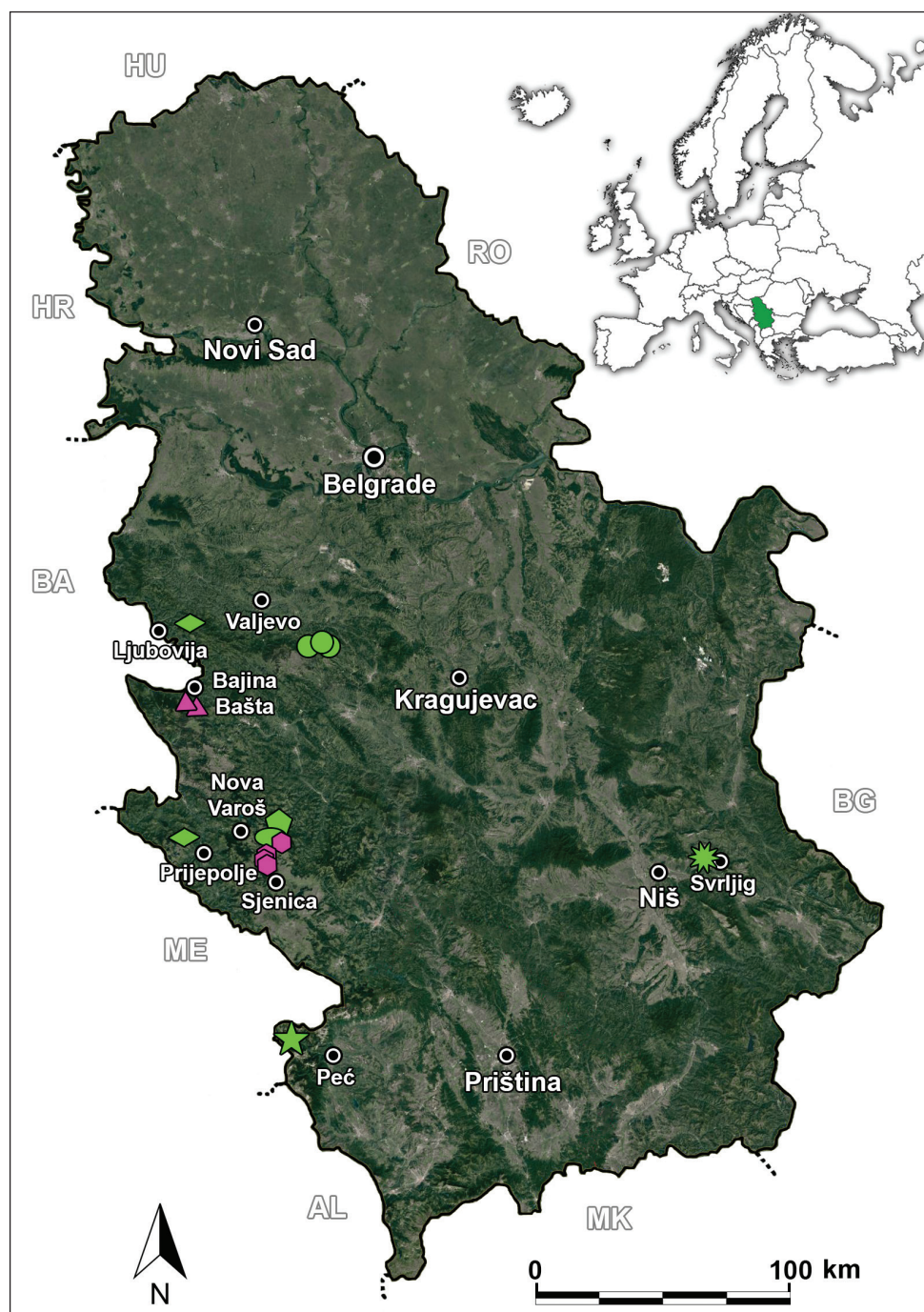


Figure 9. A map of Serbia with sites where *Bihartrechus* (in lime green colour) and *Duvalius* s. str. taxa (in pink colour) were recorded **circle** *D. (D.) suvoborensis* **ellipse** *D. (D.) suvodoensis* **hexagon** *D. (B.) reufi* **pentagon** *D. (D.) javorensis* **rhombus** *D. (D.) sturanyi sturanyi* **star** *D. (D.) leonhardi matejkai* **sun** *D. (D.) bolei* **triangle** *D. (B.) semecensis tarensis* ssp. nov. **AL** Albania **BA** Bosnia and Herzegovina **BG** Bulgaria **HR** Croatia **HU** Hungary **ME** Montenegro **MK** North Macedonia **RO** Romania.

Description. Medium-sized. TL 4.31–4.67 mm (HT 4.51 mm), L 4.83–5.18 mm.

Habitus: Body elongate. Colour reddish-brown. Legs and palpi paler.

Integument: Smooth, lustrous, both head and pronotum with a distinct isodiametric microsculpture, while microsculpture of elytra with both isodiametric and transverse meshes.

Head: Relatively large, around as long as 1/6 of TL, rounded (Fig. 2), distinctly narrower than pronotum, glabrous. Genae rounded. Neck well-developed. HL 0.70–0.75 mm (HT 0.72 mm), HW 0.80–0.87 mm (HT 0.82 mm), HL/HW 0.85–0.88 (HT 0.87). Frontal furrows complete, deep, reaching neck constriction. Two pairs of supraorbital setae present, anterior pair situated at level of reduced eyes, posterior pair near frontal furrows. Mandibles relatively long and thin, sharply pointed, right one with a retinaculum. Labrum emarginate, with three pairs of setae. Clypeus with two pairs of setae. Eyes are strongly reduced, lenticular, whitish, darkly bordered (Fig. 3), composed of a few (5–8) ommatidia. Antennae are long and slender, densely pubescent, reaching almost half of elytral length when stretched backward. Antennomere 3 longest, followed by antennomere 11, antennomere 2 shortest (A2L/A2W 2.40), scape and antennomeres 4–10 nearly equally long, A10L/A10W 2.67–2.72.

Thorax: Pronotum glabrous, transverse, cordiform (Fig. 2), slightly wider than long, with maximum width at anterior fourth, at anterior margin wider than at pronotal base. PL 0.84–0.90 mm (HT 0.90 mm), PW 0.99–1.07 mm (HT 1.06 mm), PL/PW 0.84–0.85 (HT 0.85). Lateral margins rounded anteriorly, sinuated before hind angles. Anterior angles are rounded and obtuse, while posterior angles are sharp and pointed. Lateral furrows are wide and deep, with two pairs of setae. Anterolateral pair of setae situated in the anterior fifth of pronotal length, basolateral pair of setae before hind angles. Median furrow is well-developed, deep, visible on almost whole pronotum. Disc weakly convex.

Elytra: Elongate, sub-oval, glabrous, with maximum width at the middle, apically rounded (Fig. 2). EL 2.46–2.87 mm (HT 2.70 mm), EW 1.63–1.80 mm (HT 1.79 mm), EL/EW 1.51–1.60 (HT 1.60). Elytral base oblique. Shoulders rounded, sloped. Scutellum small, sub-triangular, with one pair of scutellar setae. Elytral striae 1–5 well-developed, deep, outer striae reduced to rows of foveae. Punctuation on striae dense and deep. Elytral stria 3 with two discal setae and one apical seta. First discal seta slightly below the level of second humeral seta. Second discal seta around the middle of elytra or slightly above. Intervals are somewhat convex (inner intervals more than outer ones). Disc weakly convex, almost flattened. The umbilicate series consists of eight setae on each elytron (four humeral, two median and two apical), while the humeral group of umbilicate pores is aggregated.

Legs: Long, slender, densely pubescent (Fig. 2), each protibia dorsally with a deep longitudinal fissure. The first two tarsomeres of male protarsi are distinctly dilated and toothed at the internal margin. The first male protarsomere is longer than wide. Tarsal claws are long and slender, pointed at the apex.

Abdomen: Ventrites 4–6 and anal ventrite are glabrous, each with one pair of setae posteriorly. Male abdominal sternite IX (urite) large, sub-triangular (Fig. 7).

Male genitalia: Aedeagus (Figs 4, 5) moderately long, AEL 0.59 mm (HT 0.59 mm). The median lobe is regularly curved and gradually narrowed apically in

lateral view (Fig. 4), while almost straight, sub-parallel, with a rounded apex in dorsal view (Fig. 5). The basal bulb is relatively large (Figs 4, 5) and rounded in lateral view (Fig. 4). The copulatory piece is short, wide, unifold, in form of a shallow gutter with a longitudinal projection, rounded distally and shallowly incised proximally, weakly sclerotized except its edges and longitudinal projection (Fig. 6). The parameres are thin, each shorter than a half of aedeagus length, with four thick apical setae (Figs 4, 5).

Female genitalia: As presented in Fig. 8. Gonocoxites IX are relatively short and wide, curved, gradually narrowed apically, and basally joined with rounded, somewhat elongate gonosubcoxites IX.

Geographic distribution. So far known only from a cave and a pit, both situated on Mt. Tara (see the Type material). We assume that the subspecies could inhabit other caves and pits, as well as MSS on the same mountain and in its surroundings.

Bionomy and habitat. All specimens of *D. (B.) semecensis tarensis* ssp. nov. were collected by pitfall traps, both from the floor and walls in the innermost part of an unnamed cave in the village of Solotuša, and at the bottom of the pit 4-1-3-27 in the village of Kaluđerske Bare, in both cases in total darkness, at high humidity, and with the occurrence of trickling water. Pit 4-1-3-27 is the type locality of two additional subterranean beetle taxa – the leiodids *Pholeuonopsis (Pholeuonopsis) tarensis* Ćurčić & Pavićević, 2018 (Ćurčić et al. 2018b) and *Proleonhardella (Proleonhardella) tarensis* Ćurčić & Pavićević, 2021 (Ćurčić et al. 2021).

Duvalius (Biharotrechus) semecensis semecensis Winkler, 1926

Figs 10, 11

Type material. Holotype (by monotypy): male (NHMV) labeled as follows: „Höhle a. d. Semeč plan., Weirather“, with no date provided (white label, handwritten) / „*Duvalius semecensis* Wnl.“ (white label, handwritten) / „Holotypus *Duvalius (Biharotrechus) semecensis* Winkler, 1926 Ćurčić det. 2021“ (red label, printed) (Fig. 10).

Remarks. For the purpose of comparisons, we studied one male specimen of *D. (B.) semecensis* borrowed from the NHMV, labeled as „Höhle a. d. Semeč plan. Weirather / *Duvalius semecensis* Wnl.“ (Figs 10–13), but with no red type label added. The handwriting on both white labels appears to be that of Albert Winkler (Figs 12, 13). According to the available data (H. Schillhammer, pers. comm.), all red type labels in the collection of Albert Winkler that belong to the NHMV have been added subsequently. It seems that Winkler didn't add red type labels for his type specimens. In the original description of *D. (B.) semecensis*, it was indicated that Augusta Weirather collected one male specimen in an unnamed cave on Mt. Šjemeč in eastern Bosnia and Herzegovina (Winkler 1926). As the type series consists of only one male specimen with the same locality data, we designate the male specimen from the NHMV as the HT by monotypy of *D. (B.) semecensis*. According to Pretner (2011), who decoded the list of the cave and above-ground collecting sites visited by Weirather, the right name of the type locality of this taxon is the Vrteljka Cave, which is situated in the village



Figures 10–13. *Duvalius* (*Biharotrechus*) *semecensis semecensis* from the Vrteljka Cave, village of Đipi, near the town of Višegrad, Mt. Sjemeć, eastern Bosnia and Herzegovina **10** HT male, habitus, dorsal aspect **11** HT male, head, lateral aspect **12** locality data label of HT male **13** taxonomic name label of HT male. Scale bars: 2 mm (**10**); 0.5 mm (**11**).

of Đipi (43°44'35"N, 19°11'2"E) on Mt. Sjemeć, near the town of Višegrad, eastern Bosnia and Herzegovina. As we noted certain morphological intraspecific differences between the HT of *D. (B.) semecensis* from the NHMV and *D. (B.) semecensis tarensis* ssp. nov., the need of establishing nominotypical subspecies [*D. (B.) semecensis semecensis*] of *D. (B.) semecensis* has arisen.

After reading both the original description of *D. (B.) semecensis* by Winkler (1926) and its subsequent description by Jeannel (1928) and a careful examination of the HT of *D. (B.) semecensis*, we realized that some data on the morphology of the specimen listed in the previous literature do not correspond with the features of the observed male specimen. Namely, Jeannel (1928) reported that the head of *D. (B.) semecensis semecensis* is remarkably narrow, longer than wide, while Winkler (1926) didn't mention this in the description. The former author claimed that its pronotum is narrow, not wider than long, while the second author wrote that its pronotum is slightly wider than long. Both authors said that both discal setae are situated in fourth elytral intervals. Additionally, Jeannel's drawing of the specimen (Jeannel 1928) is not perfect as it doesn't reflect its true shape (especially the head). Indeed, in the studied HT male, both head and pro-

notum are transverse, and the second discal seta is in the third stria (as was drawn by Jeannel 1928) (Table 1, Fig. 10). For these reasons, we decided to redescribe the HT of *D. (B.) semecensis semecensis* and to add here additional data on its morphology.

Redescription. Medium-sized. TL 4.17 mm, L 4.40 mm.

Habitus: Body elongate. Colour reddish-brown. Legs and palpi paler.

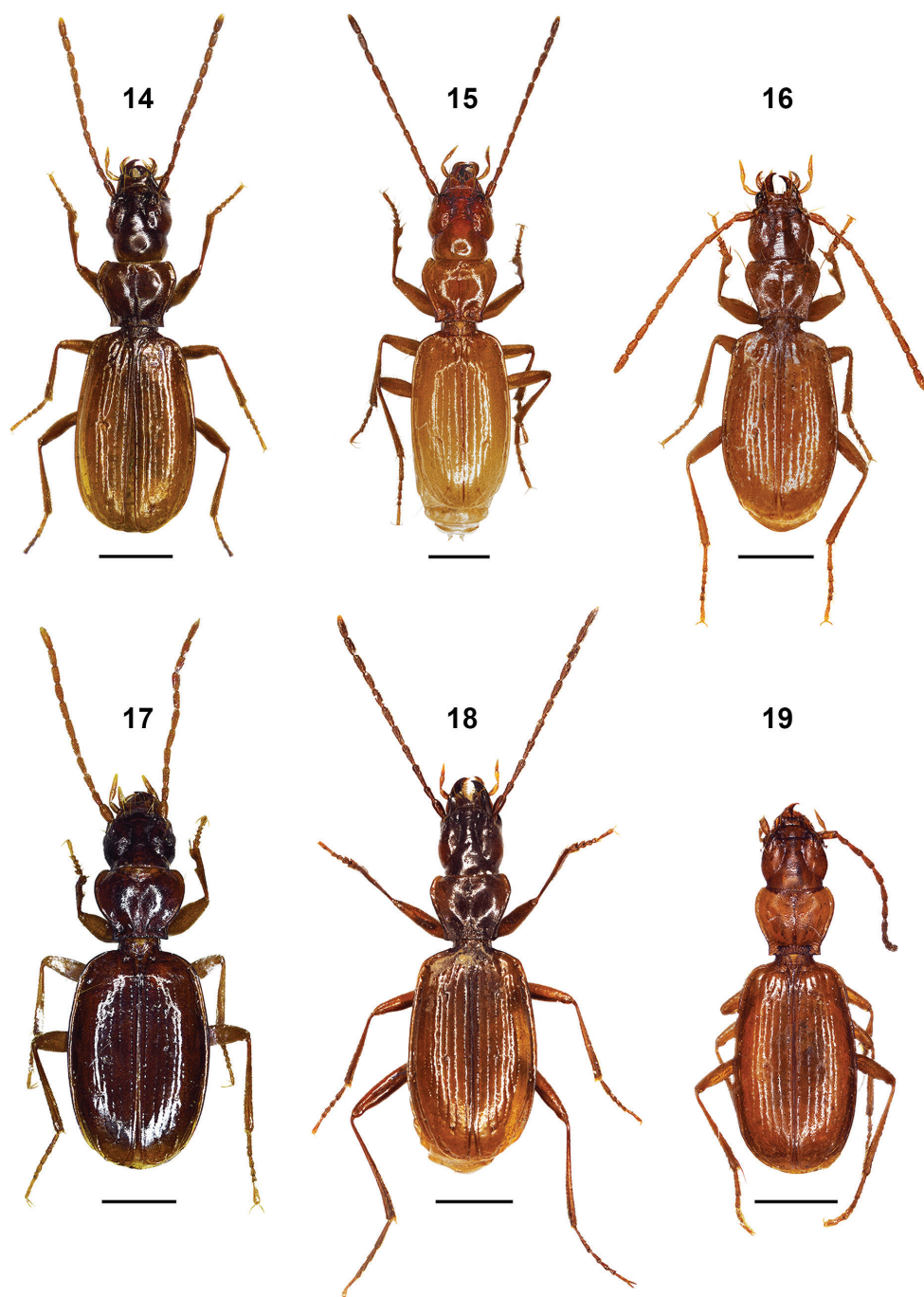
Integument: Smooth, lustrous, head with a distinct isodiametric microsculpture, while microsculpture of both pronotum and elytra possesses both transverse and isodiametric meshes.

Head: Relatively large, around as long as 1/6 of TL, rounded (Fig. 10), narrower than pronotum, glabrous. Genae are rounded. Neck well-developed. HL 0.67 mm, HW 0.79 mm, HL/HW 0.85. Frontal furrows complete, deep, reaching neck constriction. Two pairs of supraorbital setae present, the anterior pair situated at the level of reduced eyes, while the posterior pair is near frontal furrows. Mandibles are thin, relatively long, sharply pointed, right one with a retinaculum. Labrum is deeply emarginate, with three pairs of setae. Clypeus is with two pairs of setae. Eyes are strongly reduced, oval, whitish, darkly bordered (Fig. 11), composed of 1–2 ommatidia. Antennae thin, long, haired, reaching almost half of elytral length when stretched backward. Antennomere 11 longest, followed by equally long antennomeres 1 and 3, antennomere 2 shortest (A2L/A2W 1.89), antennomeres 4 and 6–10 of similar length, antennomere 5 somewhat longer, A10L/A10W 2.60.

Thorax: Pronotum glabrous, transverse, cordiform (Fig. 10), slightly wider than long, with maximum width slightly before anterior third, at anterior margin wider than at pronotal base. PL 0.83 mm, PW 1.00 mm, PL/PW 0.83. Lateral margins rounded anteriorly, sinuated before hind angles. Anterior angles are rounded and obtuse. Posterior angles are sharp and pointed. Lateral furrows are relatively narrow and shallow, with two pairs of setae. Anterolateral pair of setae situated slightly before the anterior third of pronotal length, basolateral pair of setae before hind angles. Median furrow is well-developed, deep and long. Disc weakly convex.

Elytra: Elongate, sub-ovate, glabrous, with maximum width slightly after middle, apically rounded (Fig. 10). EL 2.56 mm, EW 1.55 mm, EL/EW 1.65. Elytral base oblique. Shoulders are somewhat prominent, obtuse and sloped. Scutellum is small, sub-triangular, with one pair of scutellar setae. Elytral striae 1–5 well-developed, deep, outer striae reduced to rows of foveae. Punctuation on striae dense and deep. First discal seta is in fourth interval, second discal seta is in third stria, while apical seta is positioned at place where second and third striae coalesce, slightly below the level of second humeral seta. First discal seta slightly before the level of third humeral seta. Second discal seta after the middle of elytra. Intervals are slightly convex (inner intervals more than outer ones). Disc relatively convex. The umbilicate series consists of eight setae on each elytron (four humeral, two median and two apical), while the humeral group of umbilicate pores is aggregated.

Legs: Elongate, thin, densely pubescent (Fig. 10), each protibia dorsally has a deep longitudinal fissure. The first two protarsomeres in the male are distinctly dilated and



Figures 14–19. Habitus (dorsal aspect) of the taxa of *Biharotrechus* and *Duvalius* s. str. from Serbia **14** *D. (B.) reufi* **15** *D. (D.) bolei* **16** *D. (D.) javorensis* **17** *D. (D.) sturanyi sturanyi* **18** *D. (D.) suvoborensis* **19** *D. (D.) suvodolensis*. Scale bars: 1 mm.

toothed at the internal margin. The first male protarsomere is longer than wide. Tarsal claws are long, thin, pointed apically.

Abdomen: Ventrites 4–6 and anal ventrite are glabrous, each with one pair of setae posteriorly. Male abdominal sternite IX (urite) missing, previously extracted from the male specimen.

Male genitalia: Aedeagus missing, previously extracted from the male specimen.

Female genitalia: Unknown.

Geographic distribution. So far known from its type locality (Vrteljka Cave) situated on Mt. Sjemeć. Eric Quéinnec (pers. comm.) collected specimens of the same taxon in MSS in the surroundings of the type locality, at both sides of the Drina River, close to the town of Višegrad in eastern Bosnia and Herzegovina.

Discussion

The geographic range of the trechine genus *Duvalius* is large. It ranges from the Iberian Peninsula in the west to China in the east, as well as from central Europe in the north to northern Africa in the south. Its highest diversity is observed in the Apennine and the Balkan Peninsulas, as well as in the Alps (Jeannel 1928; Belousov 2017). This genus is exceptionally diverse and highly polymorphic and includes principally small- to medium-sized taxa (Jeannel 1928; Lorenz 2005; Belousov 2017). Their pigmentation is mostly absent, but certain taxa are pigmented. Eyes can be differently developed – lacking, reduced, or functional. Most *Duvalius* representatives are associated with subterranean habitats (chiefly caves and soil) (Jeannel 1928).

The genus *Duvalius* in the Balkan Peninsula, including Serbia, contains a high number of taxa (Lorenz 2005; Belousov 2017). The validity of some recently described taxa has been widely discussed by Lohaj et al. (2013) and Ćurčić et al. (2013). Some of them were in the past placed in newly established genera (Ćurčić et al. 2001; Ćurčić et al. 2003a, 2003b), which were later synonymized (Lohaj et al. 2013). Ćurčić et al. (2013) re-erected those to the genus level. Most authors treat the genus as a monophyletic unit (Casale et al. 2013; Faille et al. 2013; Lohaj et al. 2013), as was previously established by Jeannel (1928), but the support of this branch is not yet proven (and probably depends on the taxonomic sampling used). Namely, the monophyly of different *Duvalius* subgenera was not supported so far (Faille et al. 2013) and the organization within this group remains to be refined. Eric Quéinnec (pers. comm.) considers the genus *Duvalius* monophyletic and probably a sister group to the genus *Anophthalmus* Sturm, 1844, but thinks that the current subdivision of *Duvalius* into subgenera will probably be debatable as the boundaries of subgenera are not yet clear. However, Ćurčić et al. (2013) are of opinion that *Duvalius* contains a number of genera based on the heterogeneity of morphological features of species ascribed to the genus. It is very important to elucidate the phylogenetic position of *Duvalius* subgenera and species groups, as well as of the related genera in the Balkans in the future because phylogenetic relationships among different taxa of *Duvalius* in this area still remained

unclear. It is essential to conduct further molecular phylogenetic studies, which are independent of the subjectivity of taxonomists (contrary to the classical morphological and morphometric observations that depend on the subjective view of taxonomists).

According to morphological features, *Duvalius semecensis tarensis* ssp. nov. is provisionally ascribed to *Biharotrechus* Bokor, 1922 rather than to *Duvalius* s. str. as it predominantly shares morphological characters specific to the former subgenus. This might also concern *D. (D.) javorensis*, *D. (D.) suvoborensis* and *D. (D.) suvodolensis*, which seem to form a homogeneous group of closely related species, but it must be first supported by morphological analyses of the copulatory piece [the case of *D. (D.) suvodolensis*] and desirably by molecular studies. According to the shape of the copulatory piece, the new subspecies is close to *Duvalius* str. representatives. Namely, lateral phaneres in *D. (B.) semecensis tarensis* ssp. nov. are reaching the apex of the copulatory piece, while these structures in *Biharotrechus* spp. are short (Jeannel 1928), indicating that the subgenus *Biharotrechus* has very debatable morphological characters and is probably questionable.

In total, seven taxa of *Duvalius* s. str. and one taxon of *Biharotrechus* currently occur in Serbia (Fig. 9). As indicated in the differential diagnosis, *D. (B.) semecensis tarensis* ssp. nov. is most closely related to *D. (B.) semecensis semecensis* (inhabiting eastern Bosnia and Herzegovina), *D. (B.) reufi* (from southwestern Serbia), as well as to *D. (D.) suvoborensis*, *D. (D.) javorensis* and *D. (D.) suvodolensis* (inhabiting western and southwestern Serbia). Jeannel (1928) placed *D. (B.) semecensis* into the “*Duvalius pilifer*” species group (anophthalmous, convex *Biharotrechus* spp. with a deep fissure on the protibia, living in the Dinarides and the Hellenides), but it seems that the former taxon is not related to the “*D. pilifer*” group, which contains species with very particular morphology.

Duvalius (D.) suvoborensis, *D. (D.) javorensis* and *D. (D.) suvodolensis* likely belong to a separate group of species as they share numerous morpho-anatomical features (reduced eyes, complete and deep frontal furrows, smooth genae, the specific position of the humeral setae and the similar shape of the copulatory piece) (Pavićević and Popović 2001; Ćurčić et al. 2003a, 2003b). They were even the members of a single genus – *Javorella* S. Ćurčić, Brajković & B. Ćurčić, 2003 (Ćurčić et al. 2003a, 2003b). The species *Duvalius (Duvalius) bolei* Pretner, 1963 has a specific position within the subgenus *Duvalius* s. str. and is not closely related to *D. (B.) semecensis tarensis* ssp. nov. The former taxon is the only *Duvalius* s. str. species distributed in the Carpatho-Balkanides of Serbia. Pretner (1963) was aware of the supraspecific position of the taxon and assumed that it might belong to a separate subgenus of *Duvalius*. Based on the characteristic shape of the head, the absence of longitudinal furrows on the fore tibiae, the peculiar shape of the aedeagus and the specific form of the copulatory piece, Ćurčić and Brajković (2003) placed the taxon in the newly established genus *Curcicia* Ćurčić & Brajković, 2003.

The Serbian taxa of the subgenera *Biharotrechus* and *Duvalius* s. str. can be separated using the identification key below, followed by their catalogue, including data on their type localities, other localities in Serbia, distribution and short notes.

A provisional key for the identification of the taxa of *Biharotrechus* and *Duvallius* s. str. of Serbia

- 1 Eyes developed, both cave- and MSS-dwelling taxa, inhabiting the Dinarides in western and southwestern Serbia **2**
- Eyes completely missing, cave-dwelling species, inhabiting the Carpatho-Balkanides in southeastern Serbia ***D. (D.) bolei* Pretner, 1963**
- 2 Pigmented taxa, eyes fully functional, numerous discal setae in the third elytral striae **3**
- Depigmented taxa, eyes reduced, composed of a few ommatidia, two discal setae in the third elytral striae **4**
- 3 Body longer (TL 5.0 mm), eyes more prominent, fifth elytral striae with 1–2 discal setae, more strongly impressed, lateral pronotal margins somewhat arcuate medially ***D. (D.) leonhardi matejkai* Mařan, 1939**
- Body shorter (TL 4.5 mm), eyes less prominent, fifth elytral striae with no discal setae, less impressed, lateral pronotal margins more rounded ***D. (D.) sturanyi sturanyi* (Apfelbeck, 1904)**
- 4 Elytral base oblique, shoulders sloped, inhabiting Bosnia and Herzegovina and Serbia [*D. (B.) semecensis* Winkler, 1926] **5**
- Elytral base almost straight, shoulders elevated **6**
- 5 Antennomere 2 shorter (A2L/A2W 1.89), the maximum width of pronotum slightly before the anterior third, elytra sub-ovate, more elongate, widest below the middle, shoulders obtuse, copulatory piece with a deeper gutter and without any projection, inhabiting Mt. Sjømeć (eastern Bosnia and Herzegovina) ***D. (B.) semecensis semecensis* Winkler, 1926**
- Antennomere 2 longer (A2L/A2W 2.40), the maximum width of pronotum at the anterior fourth, elytra sub-oval, less elongate, widest at the middle, shoulders rounded, copulatory piece with a shallower gutter and a longitudinal sclerotized projection, inhabiting Mt. Tara (western Serbia) ***D. (B.) semecensis tarensis* ssp. nov.**
- 6 Head subglobose, slightly narrower than pronotum **7**
- Head elongate, distinctly narrower than pronotum **8**
- 7 Body more elongate, longer (TL 4.33–5.02 mm), head more voluminous, antennae longer, lateral elytral margins rounded, cave-dwelling species ***D. (D.) javorensis* (S. Ćurčić, Brajković & B. Ćurčić, 2003)**
- Body less elongate, shorter (TL 4.19 mm), head less voluminous, antennae shorter, lateral elytral margins arcuate, sub-parallel, MSS-dwelling species ***D. (D.) suvodolensis* (S. Ćurčić, Brajković & B. Ćurčić, 2003)**
- 8 Eyes with about 10 ommatidia, shoulders less elevated, the median lobe of aedeagus narrower and less curved in lateral view, copulatory piece sub-parallel, not incised proximally, inhabiting Mt. Suvobor ***D. (D.) suvoborensis* Pavićević & Popović, 2001**
- Eyes with no ommatidia, shoulders more elevated, the median lobe of aedeagus wider and more curved in lateral view, copulatory piece triangular, deeply incised proximally, inhabiting the Pešter Plateau and Mt. Javor ***D. (B.) reufi* Pavićević & Popović, 2003**

An annotated catalogue of the taxa of *Biharotrechus* and *Duvalius* s. str. from Serbia

1. *Duvalius (Biharotrechus) semecensis tarensis* ssp. nov. (Fig. 2)

Type locality: An unnamed cave, village of Solotuša, near the town of Bajina Bašta, Mt. Tara, western Serbia.

Other localities in Serbia: Pit 4-1-3-27, village of Kaluđerske Bare, near the town of Bajina Bašta, Mt. Tara, western Serbia.

Distribution: Serbia, Mt. Tara (cave-dwelling).

2. *Duvalius (Biharotrechus) reufi* Pavićević & Popović, 2003: 195 (*Duvalius*) (Fig. 14)

Type locality: Ušak Cave System, village of Ušak, near the town of Sjenica, Pešter Plateau, southwestern Serbia.

Other localities in Serbia: Tubića Pećina Cave, village of Tubići, near the town of Sjenica, Pešter Plateau, southwestern Serbia; Baždarska Pećina Cave, village of Ursule, near the town of Sjenica, Pešter Plateau, southwestern Serbia; Bukovik Cave, village of Ljepojevići, near the town of Nova Varoš, Mt. Javor, southwestern Serbia.

Distribution: Serbia, Pešter Plateau and Mt. Javor (cave-dwelling).

3. *Duvalius (Duvalius) bolei* Pretner, 1963: 144 (*Duvalius*) (Fig. 15)

Type locality: Prekonoška Pećina Cave, village of Prekonoga, near the town of Svrljig, Svrljiške Planine Mts., southeastern Serbia.

Other localities in Serbia: None.

Distribution: Serbia, Svrljiške Planine Mts. (cave-dwelling).

4. *Duvalius (Duvalius) javorensis* S. Ćurčić, Brajković & B. Ćurčić, 2003: 16 (*Javorella*) (Fig. 16)

Type locality: Pećina pod Kapilijama Cave, village of Trudovo, near the town of Nova Varoš, Mt. Javor, southwestern Serbia.

Other localities in Serbia: None.

Distribution: Serbia, Mt. Javor (cave-dwelling).

Note: Belousov (2017) erroneously added Bojan Mitić as one of the authors of this species.

5. *Duvalius (Duvalius) leonhardi matejkai* Mařan, 1939: 60 (*Duvalius*)

Type locality: Mt. Ćakor, Prokletije Mts., eastern Montenegro and southwestern Serbia.

Other localities in Serbia: None.

Distribution: Montenegro and Serbia, Mt. Ćakor (MSS-dwelling).

Note: Hlaváč et al. (2017) didn't include this subspecies in their catalogue. They presented data only on the nominotypic subspecies of *Duvalius (Duvalius) leonhardi* (Reitter, 1901). Belousov (2017) reported the presence of *D. (D.) leonhardi matejkai* for Montenegro only. As its type locality (Mt. Ćakor) is

situated on the border between Montenegro and Serbia (Province of Kosovo and Metohija), it has to be accounted for Serbia as well.

6. *Duvalius (Duvalius) sturanyi sturanyi* Apfelbeck, 1904: 136 (*Trechus*) (Fig. 17)

Type locality: By a small mountain stream, near the town of Foča, southeastern Bosnia and Herzegovina.

Localities in Serbia: Mt. Bobija, near the town of Ljubovija, western Serbia; Velika Pećina Cave, village of Babine, near the town of Prijepolje, southwestern Serbia.

Distribution: Bosnia and Herzegovina, Montenegro and Serbia (MSS- and cave-dwelling).

Note: Contrary to Hlaváč et al. (2017), it seems that Belousov (2017) wasn't aware of the data published in Nonveiller (1983) and Pavićević et al. (2012), who reported the presence of the nominotypic subspecies of *Duvalius (Duvalius) sturanyi* (Apfelbeck, 1904) at two sites in Serbia in the vicinity of the towns of Ljubovija and Prijepolje.

7. *Duvalius (Duvalius) suvoborensis* Pavićević & Popović, 2001: 28 (*Duvalius*) (Fig. 18)

Type locality: Pećina u Brezacima Cave, village of Brezaci, Rajac, near the town of Valjevo, Mt. Suvobor, western Serbia.

Other localities in Serbia: Mala Bezdanić Pit, Rajac, town of Valjevo, Mt. Suvobor, western Serbia; Mokra Pećina Cave, Ravna Gora, town of Valjevo, Mt. Suvobor, western Serbia.

Distribution: Serbia, Mt. Suvobor (cave-dwelling).

Note: Belousov (2017) wrote that this species was described in 2003, but actually it happened in 2001. Aside its type locality, we report here its records from two additional cave sites situated on Mt. Suvobor.

8. *Duvalius (Duvalius) suvodolensis* S. Ćurčić, Brajković & B. Ćurčić, 2003: 113 (*Javorella*) (Fig. 19)

Type locality: Valley Suvodol, village of Debelja, near the town of Nova Varoš, Mt. Javor, southwestern Serbia.

Other localities in Serbia: None.

Distribution: Serbia, Mt. Javor (MSS-dwelling).

Note: Belousov (2017) mentioned that the species lives in Armenia, but it inhabits Serbia only.

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Three new troglobitic *Coarazuphium* (Coleoptera, Carabidae, Zuphiini) species from a Brazilian hotspot of cave beetles: exploring how the environmental attributes of caves drive ground-beetle niches

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Abstract

Three new species of troglobitic beetles of the genus *Coarazuphium* are described from specimens collected in iron ore caves in the Flona de Carajás in Brazil, doubling the number of known species for the Carajás region. The new species of *Coarazuphium* are morphologically similar to the already described species from the same region and are distributed in a small geographic range. From all *Coarazuphium* species of the region, including the new ones, two stand out, *C. spinifemur* and *C. xingu* **sp. nov.**, which are the smallest *Coarazuphium* species. Both species have shorter legs and antennae when compared to the others. The main characteristic that differentiates *C. xikrin* **sp. nov.** and *C. kayapo* **sp. nov.** from the other two species from the Carajás region, *C. tapiaguassu* and *C. amazonicum*, is that the new species have more numerous setigerous punctures dorsally on the head. With the three new species added to the six already described congeners, the area of intense mining of the Carajás region includes the highest diversity of obligatory cave-dwelling beetles in Brazil, representing a hotspot of cave beetles. *Coarazuphium xikrin* **sp. nov.** and *C. amazonicum* co-occur in some of the caves of the Carajás region, which is possible due to putative

niche differentiation between the species. These findings highlight the importance of maintaining legal provisions that ensure the preservation of caves, especially those most relevant regarding physical and biotic aspects, which is crucial for the conservation of Brazilian subterranean biodiversity.

Keywords

Amazon rainforest, Brazil, cave-restricted, endemic, iron ore, outlying mean index, sympatric species

Introduction

The genus *Coarazuphium* Gnaspini, Vanin & Godoy, 1998 is currently represented by 14 species in the Neotropics. All species of the genus are subterranean as they are found in distinct underground strata. Thirteen of the 14 species are troglobitic (i.e., restricted to the cave environment) and endemic to Brazilian caves (Pellegrini et al. 2022). Only one species, *C. whiteheadi* Ball & Shpeley, 2014, is non-troglobitic and occurs in hypogeic habitats in the mountains of Oaxaca, Mexico. Almost 70% of *Coarazuphium* species are known from only one or two caves close to each other, which indicates their narrow endemism [*C. tessai* (Godoy & Vanin, 1990), *C. bezerra* Gnaspini, Vanin & Godoy, 1998, *C. cessaima* Gnaspini, Vanin & Godoy, 1998, *C. formoso* Pellegrini & Ferreira, 2011, *C. caatinga* Pellegrini & Ferreira, 2014, *C. ricardoi* Bená & Vanin, 2014, *C. spinifemur* Pellegrini & Ferreira, 2017, *C. lundi* Pellegrini, Ferreira & Vieira, 2020, *C. auleri* Pellegrini & Vieira, 2021 and *C. bambui* Pellegrini & Vieira, 2022].

Most *Coarazuphium* species are associated with caves in carbonate formations (Pellegrini and Ferreira 2011). The only known species from iron ore caves are *C. spinifemur*, *C. tapiaguassu* Pellegrini & Ferreira, 2011 and *C. amazonicum* Pellegrini & Ferreira, 2017. The last two mentioned species have a broader distribution in different caves. The lower endemism in iron ore caves is a remarkable characteristic since most *Coarazuphium* species are rare, with a few occurrences or observations (Pellegrini et al. 2020). All *Coarazuphium* species associated with iron ore caves are endemic to the Carajás Mineral Province (southeastern Amazon, Brazil). This area possesses rich mineral resources and is currently considered a cave biodiversity hotspot with many endemic or threatened species and several undescribed taxa (Jaffé et al. 2016). So, it is a great challenge to balance the conservation of endemic or threatened species with mining activities and the interests of the local human population of the Carajás region (Jaffé et al. 2016).

Troglobitic species are target taxa for conservation and can guarantee total protection to caves depending on their rarity and spatial distribution pattern (Brasil 2017). The first step towards effective conservation of a given cave species is to ensure that the species is known to science and to determine its distribution throughout the caves and habitat preferences. Thus, understanding species' habitat preferences and niche occupation are essential information to build a reliable conservation plan to protect the species and their habitats (Barlas and Yamaç 2019). However, there are little information available about the environmental conditions and natural history of *Coarazuphium*. Most of the information on *Coarazuphium* species came from specimens gathered from zoological collections, and the only available data on these species is represented by their taxonomic descriptions.

On the other hand, the ferruginous caves from the Carajás National Forest database offer a unique opportunity to study the habitat preferences of these endemic and threatened species (Jaffé et al. 2018). The newly discovered species, *Coarazuphium xikrin* sp. nov., has a broad occurrence in Carajás caves, overlapping the distribution of *C. amazonicum*. In this study, we tested the hypothesis that niche differentiation makes the coexistence of these *Coarazuphium* species possible. Evidence supports our hypothesis that in a habitat with scarce resources the coexistence of ecologically similar species is possible when traits exhibit subtle differences (Violle et al. 2011). The absence of such differences may result in competitive exclusion of recently diverged species (Webb et al. 2002). Thus, niche differentiation would reduce the interspecific competition promoting the species coexistence (Dayan and Simberloff 2005; Mayfield and Levine 2010). In particular, caves possess harsh environmental conditions that works as a highly selective ecological filter for the species pool (Mammola et al. 2016). The coexistence of *Coarazuphium xikrin* sp. nov. and *C. amazonicum* offers the opportunity to investigate the niche differentiation among them. Therefore, the main purpose of the present study is to describe three new species of *Coarazuphium* to science from Brazilian iron ore caves in the Carajás Mineral Province. Additionally, we performed the ecological niche modeling between two of the most abundant *Coarazuphium* species with co-occurrence in the caves of Carajás. Finally, we provide a key to aid species identification within the genus *Coarazuphium*.

Materials and methods

Study area

The three new species described in this work occur in caves in the eastern region of the Amazon Forest in the state of Pará, Brazil (Figs 2, 4–7). Two of them are associated with ferruginous caves in the Carajás National Forest (Flona de Carajás) – one in the Serra Norte area and the other in the Serra Sul area. The National Forest of Carajás encompasses approximately 411 thousand hectares and includes parts of the municipalities of Parauapebas, Canaã dos Carajás, and Água Azul do Norte. The National Forest of Carajás is a mosaic of protected areas forming a continuous area of 1.31 million hectares of preserved forests (Rolim et al. 2006) surrounded by cattle farmland (Campos and Castilho 2012; Martins et al. 2012). The park area mainly comprises forest formations (ombrophilous or seasonal), with only 5% consisting of rocky/rupestrian fields, which develop on the laterite plates (crusts) of high areas of the region (Campos and Castilho 2012). The third new species described in the current study was found in an iron ore cave located in the municipality of São Felix do Xingu. The metavolcanic-sedimentary sequence from the São Félix Group (also of iron-ore lithology) occurs in the extreme southwest of the Carajás Domain (Valentim and Olivito 2011; Ferreira and Lamarão 2013). There are currently 88 caves registered in this region according to the Brazilian National Register of Speleological Information database – CANIE (CECAV 2021), all in iron ore formations, and the area, unlike the Carajás National Forest, is highly altered by illegal mining, farming and illegal deforestation (Mertens et al. 2002; Souza-Filho et al. 2016; Oliveira et al. 2020; Rizzo et al. 2020).

Methods

All adult specimens used in this study were obtained from the Museu de Zoologia da Universidade de São Paulo, São Paulo (MZSP) and the Coleção de Invertebrados Subterrâneos da Universidade Federal de Lavras, Lavras, Minas Gerais (ISLA). A part of the material is stored in vials containing 70% ethanol, while other specimens are dried and fixed on entomological pins. Morphological observations were made using a Stemi 508 stereomicroscope (Carl Zeiss, Germany), while measurements and photographs were obtained using an AxioCam 506 color camera (Carl Zeiss, Germany) connected to an Axio Zoom V16 stereomicroscope (Carl Zeiss, Germany).

Fine entomological pins were used to dissect insect specimens and extract male and female genitalia. Male genitalia were treated with pancreatin to clear the soft tissues. We followed procedures for female genitalia modified after Liebherr (2015). Briefly, we first removed the entire female abdomen. The abdomen was then treated with an aqueous solution of cold 10% potassium hydroxide (KOH) overnight. The female reproductive tract was placed in glycerin and isolated from other abdominal structures first by tearing off the membranous tergites followed by removal of the digestive tract and tracheal system, and finally removing the ventrites. We then mounted the female reproductive tract on glass slides in Kayser glycerol gelatin using standard procedures developed for mites (Walter and Krantz 2009). Observations and imaging of female genitalia were made using a DM750 microscope (Leica, Germany) and an Axio Zoom V16 stereomicroscope (Carl Zeiss, Germany).

All figures were edited using the Adobe Photoshop CS5 software, version 12.0. The use of terms for male and female genital structures follows Ball and Shpeley (2013) and Liebherr and Will (1998). All measurements are in millimeters (mm).

Abbreviations of body measurements and structures

A1L	scape length;
A2–4L	length of antennomeres 1–3 combined;
AL	antennal length from the base of the scape to the tip of the 10 th antennomere;
ASOS	anterior supraorbital setigerous punctures;
EL	elytral length (linear distance from the humerus to the apex);
EW	maximum elytral width (the longest linear transverse distance);
HL	head length (linear distance from the apex of the clypeus to the posterior margin of the postocciput);
HW	head width (maximum transverse distance across head, including eyes);
HWL	length of hind wings (the longest linear transverse distance);
OBL	overall body length (the sum of HL, PL, and EL);
OCS	occipital setigerous punctures;
PL	pronotum length (linear distance from the anterior margin to the posterior margin measured along the midline);

- POS** postocular setigerous punctures;
PSOS posterior supraorbital setigerous punctures;
PSUS posterior supernumerary setigerous punctures;
PW maximum pronotum width (the longest linear transverse distance) (Fig. 1).

Abbreviations of male genital structures

- GSL** genital segment length;
GSW genital segment width;
LPL left paramere length;
MLA length of median lobe of aedeagus (measured in a straight line from the basal margin to the apical margin);
OML peristial membrane length (measured in a straight line from the basal margin to the apical margin);
RPL right paramere length.

It is worth mentioning that in most Carabidae, the aedeagus has a 180° torsion in an active situation (Deuve 1993). By convention, in this paper what is called ventral is anatomically dorsal, what appears right is anatomically left, and what appears left is anatomically right.

Cave physical characteristics and organic resources

Data on the physical characteristics and organic resources of the sampled caves were obtained by consulting companies that employed standardized methods for the same set of cave attributes (for more details see the Brazilian legislation for the protection of caves:

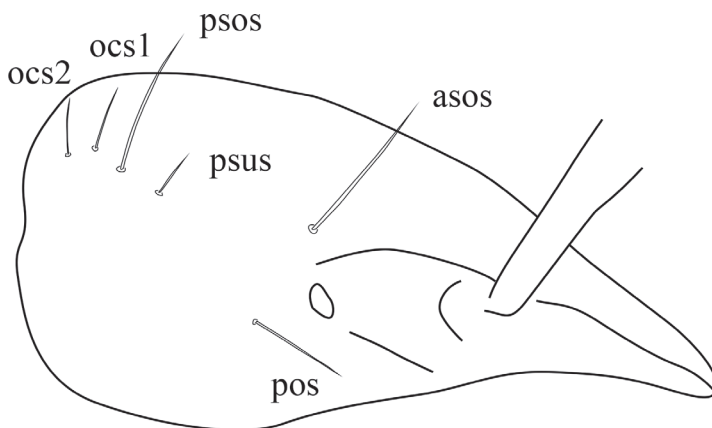


Figure 1. A schematic representation of setigerous punctures distribution on the head (lateral view) in *Coarazuphium* species. For codes interpretation see the Materials and methods section.



Federal Decree 6640/2008 and Normative Instruction MMA 02/2017) (Brasil 2008, 2017). The cave characteristics included the following data: GPS coordinates, altitude (taken at the cave entrance), horizontal projection (length), slope, area, volume (these last four parameters were obtained from the cave maps), and the presence of percolating water or water reservoirs (e.g., puddles or lakes). The organic resources recorded included the presence of plant debris, organic detritus, roots, guano, feces from vertebrates other than bats, and regurgitation balls from owls and carcasses. These organic resource variables were not evaluated separately, but were analyzed together to calculate the diversity of organic matter resources available using Shannon's diversity index.

Data analyses

To evaluate the marginal use of the available habitat by two sympatric species (*Coarazuphium amazonicum* and *C. xikrin* sp. nov.), we performed the outlying mean index (OMI). OMI is a multivariate technique that measures the distance between the mean values used and the mean values available for each environmental condition of the total sampled area (Dolédéc et al. 2000). This analysis provides information on niche marginality (average distance of the hypervolume centroid – OMI), tolerance values (niche width – Tol), and residual tolerance (variation of niche width that was not related to the studied variables – Rtol). In addition, it calculates the total inertia that estimates the influence of environmental variables on niche separation (Dolédéc et al. 2000).

The cave characteristics and organic matter diversity measures mentioned above were standardized to a mean of 0 and a standard deviation equal to 1 in order to make the scales comparable. All available adult specimens of *C. amazonicum* (27) and *C. xikrin* sp. nov. (33) were included in this analysis to improve model accuracy. The analysis was performed using the R software, version 3.6.2 (R Core Team 2019) utilizing the “ade4” package (Dray and Dufour 2007). The Monte Carlo test with 10,000 permutations was used to evaluate the significance of niche marginality and the average marginality of species (Dolédéc et al. 2000).

Figures 2–7. Geographic distribution of *C. xikrin* sp. nov., *C. kayapo* sp. nov. and *C. xingu* sp. nov. **2** location of the caves with occurrences of the new species in the state of Pará, Brazil and South America **3** *Coarazuphium kayapo* sp. nov., a living specimen **4** location of the caves (circles) where *Coarazuphium* species have been collected. Shaded regions correspond to the different highlands in the area **5** photograph of the São Félix do Xingu region, where is situated the type locality (Cave SFX-0057) of *C. xingu* sp. nov. **6** photograph of the N1N8 area from the Serra Norte highland, where most of caves with occurrences of *C. xikrin* sp. nov. are located **7** photograph of a lake found in the Serra Sul highland, where caves with occurrences of *C. kayapo* sp. nov. are located. Photo credits: Ativo Ambiental (**3**), Fundação Casa de Cultura de Marabá (**5**), Marcelo Rosa (**6**), and Robson de Almeida Zampaulo (**7**).

Results

Order Coleoptera Linnaeus, 1758

Family Carabidae Latreille, 1802

Tribe Zuphiini Bonelli, 1810

Genus *Coarazuphium* Gnaspini, Vanin & Godoy, 1998

***Coarazuphium xikrin* Pellegrini, Ferreira & Vieira, sp. nov.**

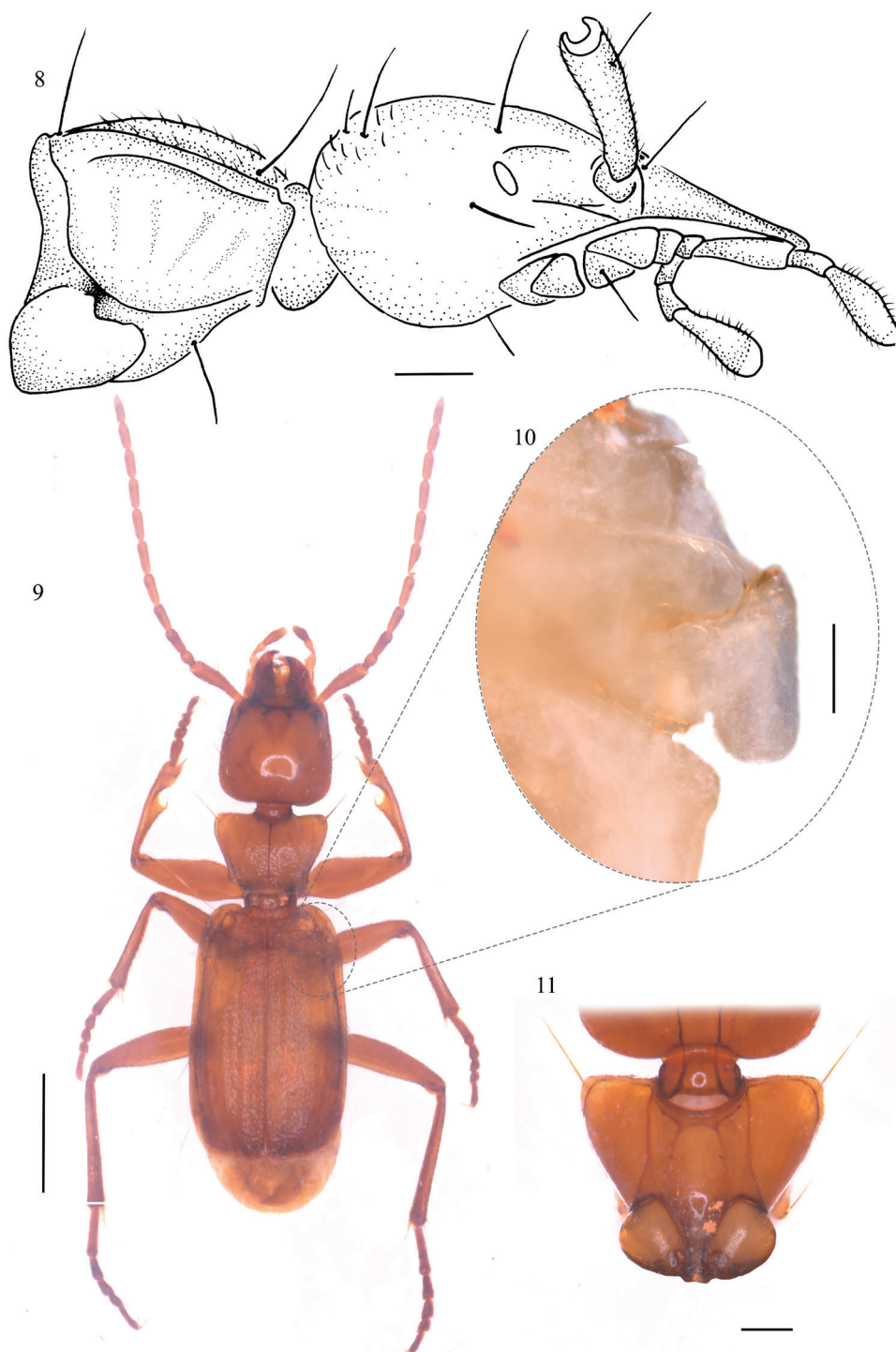
<http://zoobank.org/0BB32336-7C52-477A-A44D-7466B7C6AE7D>

Figs 8–17

Type material. *Holotype*: BRAZIL: Pará, Serra dos Carajás, Cave N1N8/N1-0020 Flona Carajás, PA, 6°01'57.7"S, 50°16'18.6"W, 639 m a.s.l., ♂, 17.VII–04.VIII.2014, Carste Company leg. (MZSP49180).

Paratypes (10 specimens). BRAZIL: Pará, Serra dos Carajás, Cave N1N8/N1-0022 Flona Carajás, PA, 6°01'57.0"S, 50°16'18.6"W, 642 m a.s.l., 1 ♀, 17.VII–04.VIII.2014, Carste Company leg. (MZSP49181); the same locality as for preceding, 1 ♂, 17.VII–04.VIII.2014, Carste Company leg. (MZSP49182); BRAZIL: Pará, Serra dos Carajás, Cave N1N8/N1-0008 Flona Carajás, PA, 6°02'19.9"S, 50°16'13.1"W, 700 m a.s.l., 1 ♀, 24.II–13.III.2015, Carste Company leg. (MZSP49183); BRAZIL: Pará, Serra dos Carajás, Cave N1N8/N1-0073 Flona Carajás, PA, 6°01'13.5"S, 50°17'17.4"W, 507 m a.s.l., 1 ♀, 02–29.IV.2015, Carste Company leg. (MZSP49184); BRAZIL: Pará, Serra dos Carajás, Cave N1N8/N1-0037 Flona Carajás, PA, 6°01'49.9"S, 50°16'27.7"W, 723 m a.s.l., 1 ♂, 24.II–13.III.2015, Carste Company leg. (MZSP49185); BRAZIL: Pará, Serra dos Carajás, Cave N1N8/N1-0016 Flona Carajás, PA, 6°01'09.7"S, 50°16'40.9"W, 531 m a.s.l., 1 ♂, 04.IX–06.X.2014, Carste Company leg. (MZSP49186); BRAZIL: Pará, Serra dos Carajás, Cave N1N8/N1-0168 Flona Carajás, PA, 6°01'16.3"S, 50°18'05.1"W, 675 m a.s.l., 1 ♂, 17.VII–04.VIII.2014, Carste Company leg. (MZSP49187); BRAZIL: Pará, Serra dos Carajás, Cave N1N8/N1-0240 Flona Carajás, PA, 6°01'18.5"S, 50°16'26.1"W, 638 m a.s.l., 1 ♀, 04.IX–06.X.2014, Carste Company leg. (MZSP49188); BRAZIL: Pará, Serra dos Carajás, Cave N1N8/N1-0101 Flona Carajás, PA, 6°01'08.7"S, 50°16'46.2"W, 541 m a.s.l., 1 ♀, 04.IX–06.X.2014, Carste Company leg. (MZSP49189); Cave N1N8/N1-0037 Flona Carajás, PA, 6°01'49.9"S, 50°16'27.7"W, 723 m a.s.l., 1 ♀, 04.IX–06.X.2014, Carste Company leg. (MZSP49190).

Additional material examined (seven specimens). BRAZIL: Pará, Serra dos Carajás, Cave N1N8/N1-0062 Flona Carajás, PA, 6°01'09.6"S, 50°16'44.4"W, 533 m a.s.l., 1 ♀, 04.IX–06.X.2014, Carste Company leg. (MZSP49191); BRAZIL: Pará, Serra dos Carajás, Cave N1N8/N1-0016 Flona Carajás, PA, 6°01'09.7"S, 50°16'41.0"W, 535 m a.s.l., 1 ♀, 04.IX–06.X.2014, Carste Company leg. (MZSP49192); BRAZIL: Pará, Serra dos Carajás, Cave N1N8/N1-0240 Flona Carajás, PA, 6°01'18.5"S, 50°16'26.1"W, 599 m a.s.l., 1 ♀, 04.IX–06.X.2014, Carste Company leg. (MZSP49193); the same locality as for preceding, 1 ♂, 02–29.IV.2015, Carste Company leg. (MZSP49194); BRAZIL: Pará, Serra dos Carajás, Cave N1N8/N1-0025 Flona Carajás, PA,



Figures 8–11. *Coarazuphium xikrin* sp. nov., external morphology **8** head and prothorax, lateral view **9** habitus, dorsal view **10** a detail of hind wings, dorsal view **11** prothorax, ventral view. Scale bars: 1.0 mm (**9**); 0.5 mm (**8**); 0.2 mm (**11**); 0.1 mm (**10**).

6°01'49.5"S, 50°16'19.8"W, 621 m a.s.l., 1 ♀, 02–29.IV.2015, Carste Company leg. (MZSP49195); BRAZIL: Pará, Serra dos Carajás, Cave N1N8/N1-0037 Flona Carajás, PA, 6°01'49.9"S, 50°16'27.7"W, 723 m a.s.l., 1 ♀, 04.IX–06.X.2014, Carste Company leg. (MZSP49196); BRAZIL: Pará, Serra dos Carajás, Cave N1N8/N1-0052 Flona Carajás, PA, 6°01'49.5"S, 50°16'19.8"W, 771 m a.s.l., 1 ♀, 24.II–13.III.2015, Carste Company leg. (MZSP49197).

Etymology. The species name honors the Xikrin ethnic group (Brazilian Indians), which live in the Carajás region. The Xikrin Indians speak the Kayapó language, which emphasizes listening and speaking. To sharpen these qualities, the Xikrin pierce, as early as infancy, the corresponding organs (ears and lips). For this ethnic group, listening is related to knowing, to acquiring knowledge. Oral communication, in turn, is a highly valued social practice for the Kayapó groups in general, who define themselves as those who speak well and beautifully. This noun should be treated as in apposition.

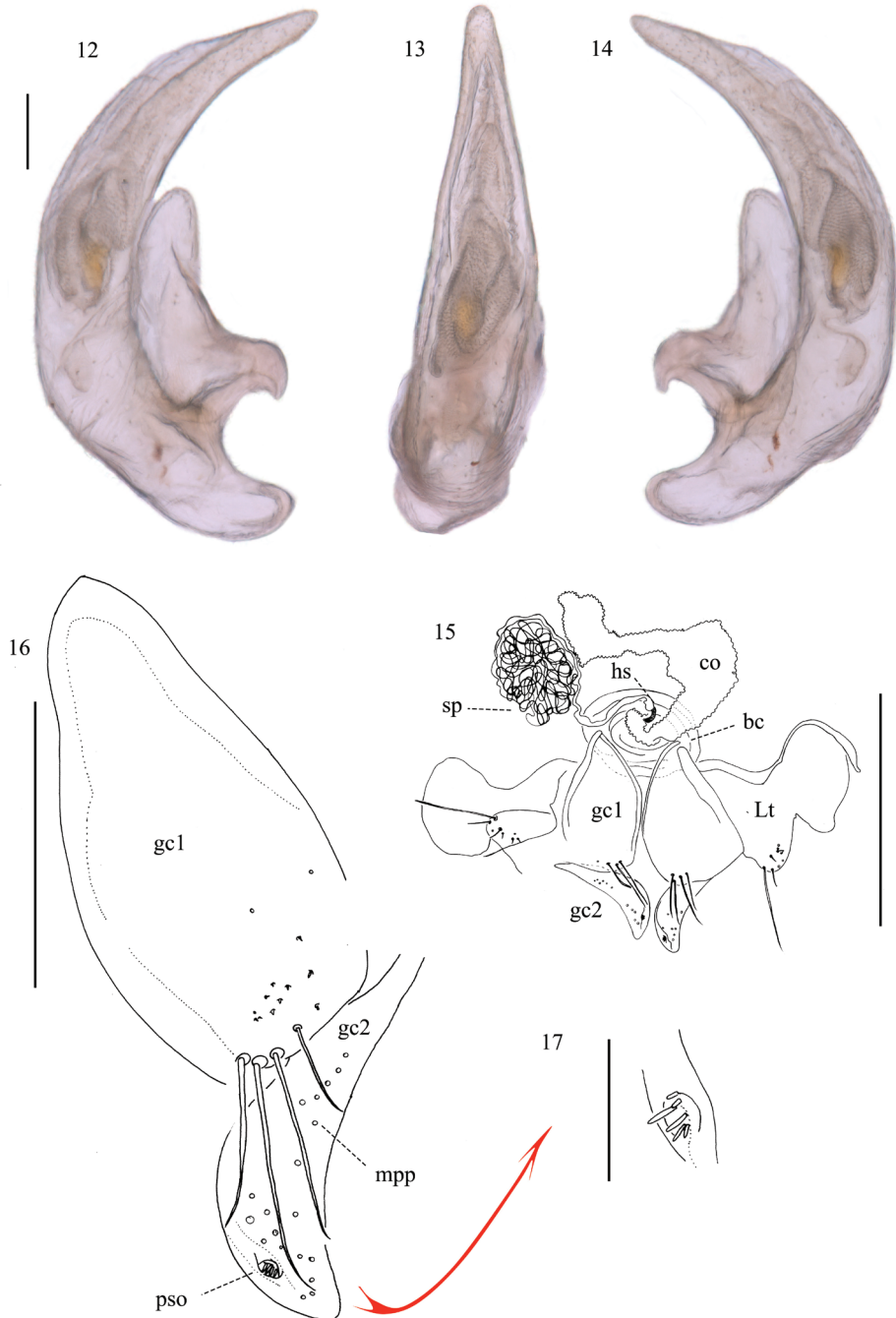
Differential diagnosis. All characteristics of *C. xikrin* sp. nov. are consistent with the description of the genus *Coarazuphium*. This species differs from all other members of the genus by the following combination of characters: elytral outline subparallel, elytra with maximum width in the posterior half, with apical margin truncate, without subapical sinuosity; location of setigerous punctures on the head dorsally: one pair of anterior supraorbital, one pair of postocular, one pair of posterior supraorbital and one pair of occipital; metafemur without a spine medially at its ventral side; antennae long, about 0.80 times as long as body length; median lobe of aedeagus about 2.81 as long as left paramere and 4.55 as long as right paramere.

Description. Size and proportions. OBL: 3.89 mm (3.82–3.99 mm ♂♂, 3.79–4.06 mm ♀♀); EW: 1.36 mm (1.27–1.39 mm ♂♂, 1.29–1.45 mm ♀♀); HW/PW: 0.99 (0.99–1.02 ♂♂, 1.04–1.09 ♀♀).

Habitus. Body with uniform pale to dark brown color (Fig. 9).

Integument. Dorsally covered with short recumbent hairs.

Head. Subtrapezoidal (Fig. 9), HW/HL: 0.93 (0.87–0.95 ♂♂, 0.89–0.98 ♀♀). Head almost as wide as pronotum. Setigerous punctures on the head dorsally: one pair of anterior supraorbital above the eyes; one pair of postocular immediately behind the eyes, laterally; one pair of posterior supraorbital posteriad the eyes; and one pair of occipital in the posterior margin of the head. There is also a pubescence evenly distributed on the head. The pubescence bristles are slightly bigger on the vertex of the head. Ventrally are four pairs of setae, three close to the gular region and the other located lateromedially (Fig. 8). Eyes reduced, depigmented, and flattened, situated laterally at the end of the genal sulcus, ommatidia are not visible at 50×. Antennae filiform and flagellar (Fig. 9), AL: 3.10 mm (3.08–3.18 mm ♂♂, 3.00–3.16 mm ♀♀), AL/PL: 4.89 (4.69–5.13 ♂♂, 4.58–4.97 ♀♀), A1L/A2–4L: 0.73 (0.75–0.86 ♂♂, 0.75–0.88 ♀♀), of almost the same length in both genders. First antennomere (scape) with a long seta distally close to the apical portion and a row of several semi-erect setae; 2nd one very short. Third antennal segment elongate, antennal segments 4–10 subequal and almost round in cross-section, except for the tip of the terminal antennomere, which is laterally flattened.



Figures 12–17. *Coarazuphium xikrin* sp. nov., male and female genitalia **12** aedeagus, left lateral view **13** aedeagus, dorsal view **14** aedeagus, right lateral view **15** female reproductive tract, dorsal view **16** gonocoxite, dorsal view **17** preapical setose organ, dorsal view **bc** bursa copulatrix **co** common oviduct **gc1** gonocoxite 1 **gc2** gonocoxite 2 **hs** helminthoid sclerite **Lt** laterotergite **mpp** marginal pit pegs **pso** preapical setose organ **sp** spermatheca **spg** spermathecal gland **spgd** spermathecal gland duct. Scale bars: 0.5 mm (**15**, **17**); 0.125 mm (**16**); 0.1 mm (**12–14**).

Prothorax. Pronotum trapezoidal, PL/PW: 0.65 (0.63–0.70 ♂♂, 0.65–0.70 ♀♀) (Figs 8, 9). Maximum pronotum width closely behind the anterior margin, which is almost as wide as the head. Anterior angle rounded. Posterior angle acute. Dorsal surface with two pairs of lateral marginal erect setae: one very long (0.8 times as long as pronotum), close to the antero-lateral angles, and the other shorter (0.5 times as long as pronotum), close to the postero-lateral angles. Prosternum with a pair of submedial setae (Figs 8, 11).

Pterothorax. Metasternum longer than wide. Metepisternum wider than long.

Elytra and hind wings. Elytra are free, with almost parallel sides (Fig. 9), EL/EW: 1.61 (1.56–0.70 ♂♂, 1.49–1.68 ♀♀). Maximum elytral width in the posterior third, EW/PW: 1.40 (1.34–1.50 ♂♂, 1.38–1.52 ♀♀). Elytral apex is truncate, not sinuate. Elytral chaetotaxy: no discal setae present; the umbilicate series of the 8th stria with seven large setae (about 0.58 as long as elytra) on each elytron, situated as follows: three close to the anterior angle, two marginal in the lateral posterior half, and two on the posterior margin. Hind wings very reduced (Fig. 10), 0.204-mm long, HWL/EL: 0.09.

Legs. Profemur 1.05 (1.02–1.13 ♂♂, 1.03–1.20 ♀♀) times as long as mesofemur and 0.75 (0.70–0.77 ♂♂, 0.68–0.77 ♀♀) times as long as metafemur. Protibia 1.20 (1.02–1.20 ♂♂, 1.02–1.19 ♀♀) times as long as mesotibia and 0.77 (0.65–0.76 ♂♂, 0.69–0.79 ♀♀) times as long as metatibia. Protibia 1.33 (1.16–1.35 ♂♂, 1.20–1.43 ♀♀) times as long as protarsus. Mesotibia 0.85 (0.89–0.98 ♂♂, 0.87–0.96 ♀♀) times as long as mesotarsus. Metatibia 0.94 (0.97–1.03 ♂♂, 0.97–1.04 ♀♀) times as long as metatarsus. First pro-, meso-, and metatarsomere each almost equal to tarsomeres 2–4 combined. Length of protibia and protarsus combined 2.48 (2.32–2.48 ♂♂, 2.28–2.56 ♀♀) times as long as pronotum, length of mesotibia and mesotarsus combined 2.56 (2.54–2.74 ♂♂, 2.36–2.69 ♀♀) times as long as pronotum, while length of metatibia and metatarsus combined 3.77 (3.56–3.78 ♂♂, 3.50–3.75 ♀♀) times as long as pronotum.

Abdomen. Ventrites 2–7 with a very fine pubescence. Seventh ventrite with a pair of short ventral setae at its posterior margin. Male genital segment triangular, GSL: 0.81 mm, GSW: 0.49 mm.

Aedeagus. Median lobe of aedeagus slightly curved ventrally and elongate, narrowed apically, apical margin rounded (Figs 12–14), MLA: 0.73 mm, OML: 0.27 mm. Left paramere subtriangular, conchoid, about twice as long as wide, LPL: 0.26 mm; right paramere styloform, about three times as long as wide, distinctly shorter than the left one, RPL: 0.16 mm.

Female reproductive tract. Ovipositor (Figs 16, 17): with a broad laterotergite; basal gonocoxite 1 longer than apical gonocoxite 2, with three and four long trichoid setae apicoventrally, respectively (from left and right gonocoxites 1); gonocoxite 2 strongly curved, falciform in lateral aspect, with slightly rounded apex, with preapical setose organ circuloid ventrally, with four nematiform setae, laterodorsal surface with many marginal pit pegs (medially, on the lateroventral surface, the marginal pit pegs are located more apically). Female genital tract totally membranous (Fig. 15). Bursa copulatrix bulbous, without any expansion in

a bursal sacculus. Apically to the bursa copulatrix is the insertion point of common oviduct, which has a sharp curve to the right basally. Spermathecal insertion is in the helmonthoid sclerite, in the junction of common oviduct and bursa copulatrix. Spermatheca is markedly elongated and slender, widening distally. Spermathecal gland duct and spermathecal gland were not visualized. No secondary spermathecal gland observed.

Distribution. The species is widely distributed in caves from plateaus known as the N1N8 area in the northwestern part of the “Serra Norte de Carajás”, although a few specimens were also found in caves located southeast of the same plateaus at location “Serra Norte de Carajás”, state of Pará, Brazil (Figs 2, 4, 6).

***Coarazuphium kayapo* Pellegrini, Ferreira & Vieira, sp. nov.**

<http://zoobank.org/9280A871-017C-4D74-B1DA-46DF83182909>

Figs 3, 18–28

Type material. Holotype: BRAZIL: Pará, Serra Sul de Carajás, Cave S11B-0177, 6°20'21.4"S, 50°30'21.0"W, 641 m a.s.l., ♂, 24.IX.2018, Ativo Ambiental leg. (ISLA 75757).

Paratype. BRAZIL: Pará, Serra Sul de Carajás, Cave S11D-0111, 6°23'48.1"S, 50°20'27.3"W, 1 ♀, 17.VII–04.VIII.2010, Carste Company leg. (MZSP49198).

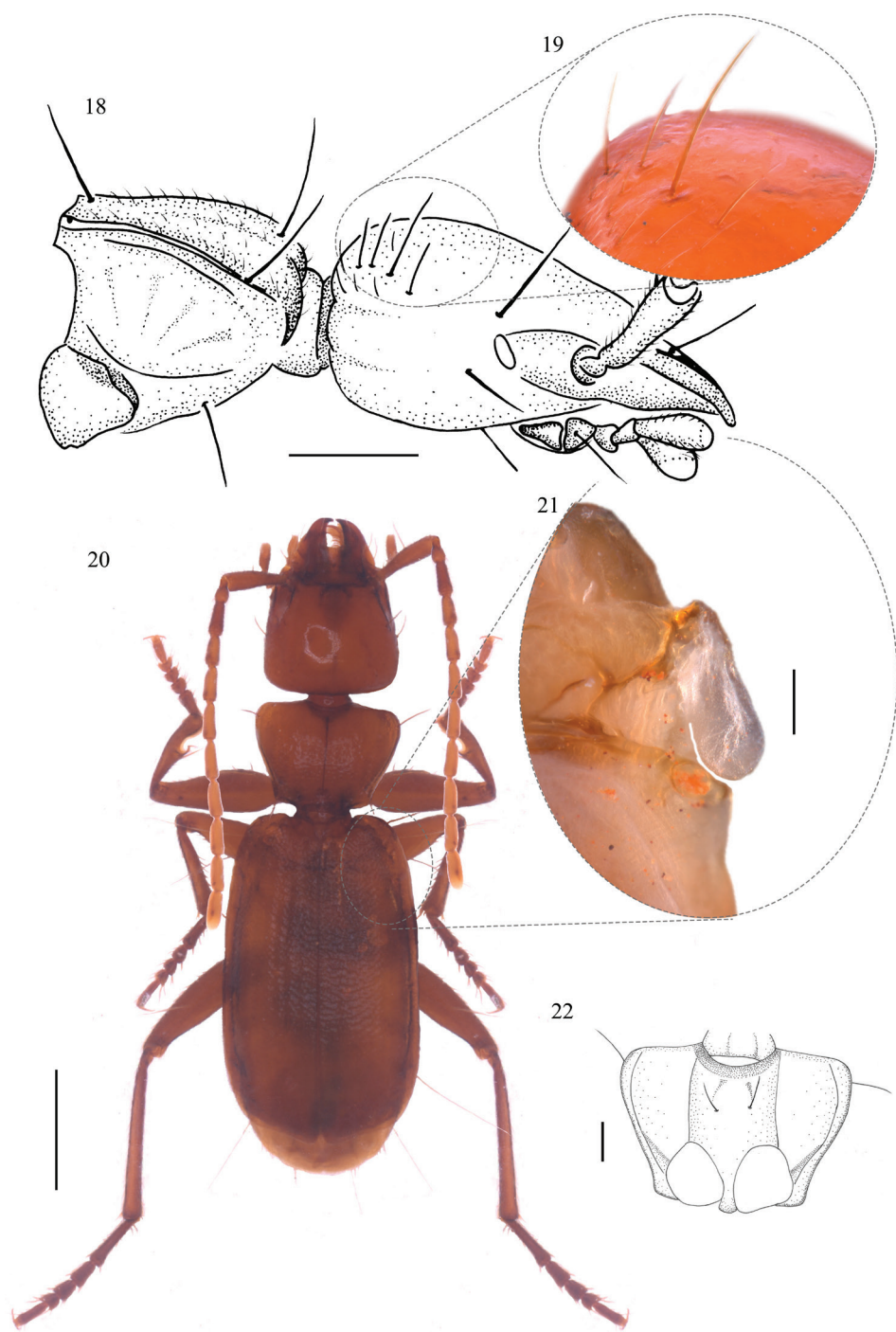
Etymology. The species name honors the Kayapó ethnic group, which is an important ethnic group of Brazilian indigenous people who live in the Amazon region. The natives do not designate themselves by this term, which was coined by neighboring groups to name them and which means “those who resemble monkeys”, which is probably due to a ritual during which the Kayapó men, dressed in monkey masks, perform short dances. The Kayapó refer themselves as the mebêngôkre, which means “the men of the hole/place of water”.

Differential diagnosis. All characteristics of *C. kayapo* sp. nov. are consistent with the description of the genus *Coarazuphium*. This species differs from all other members of the genus by the following combination of characters: elytral outline subparallel, elytra with maximum width in the posterior half, with a very slight preapical sinuosity; location of setigerous punctures on the head dorsally: one pair of anterior supraorbital, one pair of postocular and one pair of posterior supraorbital posteriad the eyes; three other pairs of setae, smaller in length, surrounding the latter setigerous punctures; head has a pubescence concentrated in the vertex margin, with long bristles; antennae long, about 0.76 times as long as body length; metafemur without a spine medially at the ventral side; median lobe of aedeagus about 2.77 as long as left paramere and 5.30 as long as right paramere.

Description. Size and proportions. OBL: 4.88 mm ♂, 4.96 mm ♀; EW: 1.61 mm ♂, 1.69 mm ♀; HW/PW: 0.93 ♂, 1.00 ♀.

Habitus. Body with uniform orange to brown color (Fig. 20).

Integument. Dorsally covered with short recumbent hairs.



Figures 18–22. *Coarazuphium kayapo* sp. nov., external morphology **18** head and prothorax, lateral view **19** a detail of fixed setae dorsally in the posterior portion of the head, lateral view **20** habitus, dorsal view **21** a detail of hind wings, dorsal view **22** prothorax, ventral view. Scale bars: 1.0 mm (**20**); 0.5 mm (**18**); 0.2 mm (**22**); 0.1 mm (**19**, **21**).

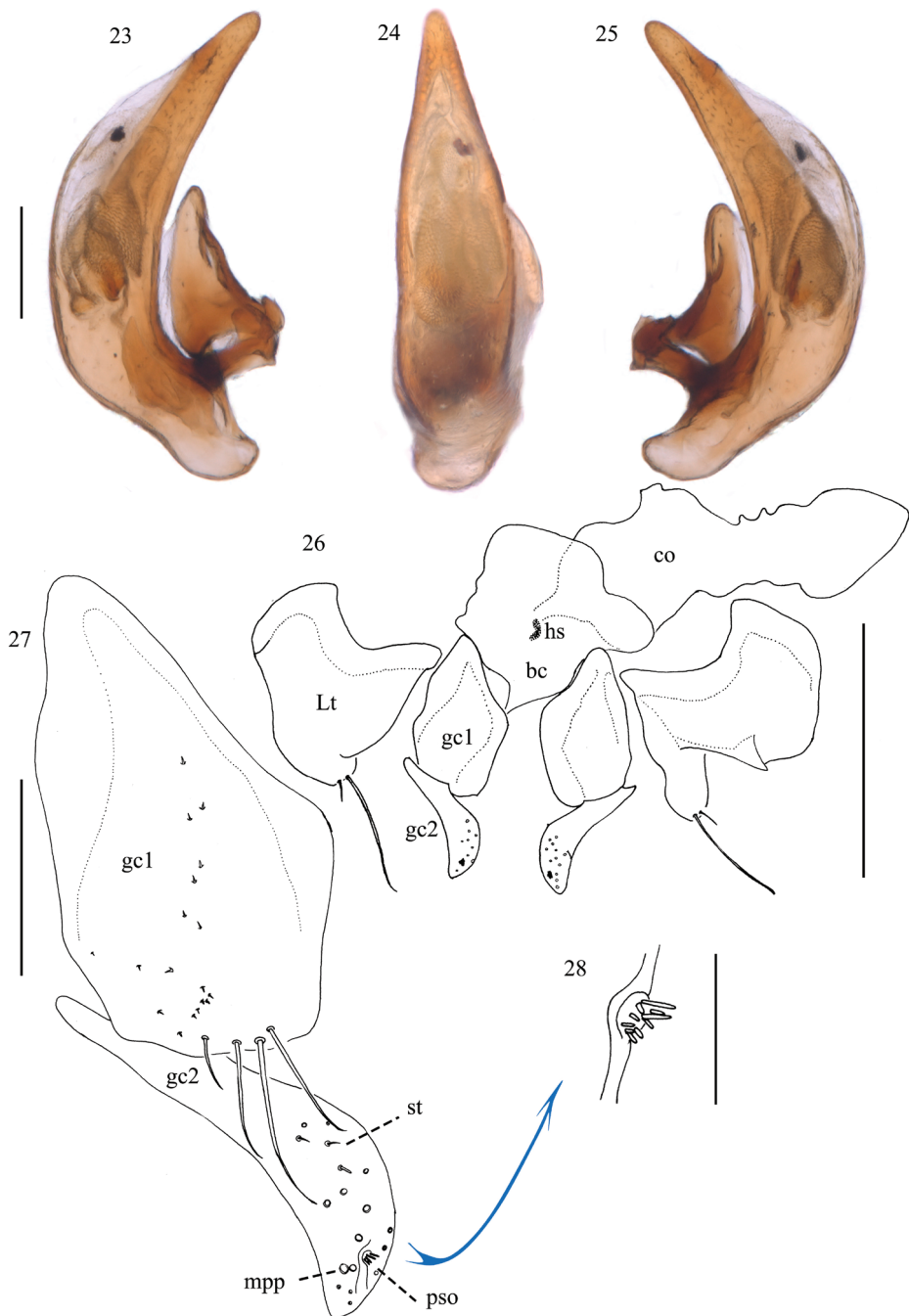
Head. Subtrapezoidal (Fig. 20), HW/HL: 0.91 ♂, 0.97 ♀. Head almost as wide as pronotum. Setigerous punctures on the head dorsally marginally to the vertex: one pair of anterior supraorbital above the eyes; one pair of postocular immediately behind the eyes, laterally; one pair of posterior supraorbital; three other pairs of setae, smaller in length, surrounding the latter setigerous punctures; among these smaller setae, there are two pairs of occipital setae; pubescence is concentrated on the vertex of the head, some of those bristles are almost of the size of medium-sized setae. Ventrally, the pubescence is sparse and the bristles are of varying size, located on the mentum, mentum suture and in the apical third of the post-gena. Associated with the post-gena, one pair of fixed setae is located more outwards apically, next to the submentum. There is also a pubescence irregular in size and position (Figs 18, 19). Eyes reduced, depigmented and flattened, situated laterally at the end of the genal sulcus, ommatidia are not visible at 50×. Antennae filiform and flagellar (Fig. 20), AL: 3.69 mm ♂, 3.59 ♀, AL/PL: 4.22 ♂, 4.03 ♀, A1L/A2–4L: 0.86 ♂, 0.88 ♀, of almost the same length in both genders. First antennomere (scape) with a long seta distally close to the apical portion, and a row of several semi-erect setae; 2nd one very short. Antennal segments 3–10 subequal and almost round in cross-section, except for the tip of the terminal antennomere, which is laterally flattened.

Prothorax. Pronotum trapezoidal, PL/PW: 0.72 ♂, 0.74 ♀ (Figs 18, 20). Maximum pronotum width closely behind the anterior margin, which is almost as wide as head. Anterior angle rounded. Posterior angle acute. Dorsal surface with two pairs of lateral marginal erect setae: one longer, close to the antero-lateral angles (about 0.43 times as long as pronotum), and the other shorter, close to the postero-lateral angles. Prosternum with a pair of submedial anterior setae (Fig. 22).

Pterothorax. Metasternum longer than wide. Metepisternum wider than long.

Elytra and hind wings. Elytra free (Fig. 20), EL/EW: 1.72 ♂, 1.67 ♀. Elytra subparallel, with maximum width in the posterior third, EW/PW: 1.33 ♂, 1.40 ♀. Elytral apex with a very slight sinuosity. Elytral chaetotaxy: no discal setae present; the umbilicate series of the 8th stria with seven large setae (about 0.43 times as long as elytra) on each elytron situated as follows: three close to the anterior angle, two marginal in the lateral posterior half, and two on the posterior margin. Hind wings are very reduced (Fig. 21), 0.275-mm long, HWL/EL: 0.10.

Legs. Profemur 1.06 (♂) and 1.03 (♀) times as long as mesofemur, and 0.71 (♂, ♀) times as long as metafemur, respectively. Protibia 0.99 (♂) and 1.17 (♀) times as long as mesotibia, and 0.69 (♂) and 0.73 (♀) times as long as metatibia, respectively. Protibia 1.18 (♂) and 1.35 (♀) times as long as protarsus, respectively. Mesotibia 0.99 (♂) and 0.90 (♀) times as long as mesotarsus, respectively. Metatibia 1.02 (♂) and 1.01 (♀) times as long as metatarsus. First pro-, meso-, and metatarsomere each almost equal to tarsomeres 2–4 combined. Length of protibia and protarsus combined 1.93 (♂) and 1.99 (♀) times as long as pronotum, length of mesotibia and mesotarsus combined 2.12 (♂) and 2.07 (♀) times as long as pronotum, while length of metatibia and metatarsus combined 3.02 (♂) and 3.10 (♀) times as long as pronotum, respectively.



Figures 23–28. *Coarazuphium kayapo* sp. nov., male and female genitalia **23** aedeagus, left lateral view **24** aedeagus, dorsal view **25** aedeagus, right lateral view **26** female reproductive tract, dorsal view **27** gonocoxite, dorsal view **28** preapical setose organ, dorsal view **bc** bursa copulatrix **co** common oviduct **gc1** gonocoxite 1 **gc2** gonocoxite 2 **hs** helminthoid sclerite **Lt** laterotergite **mpp** marginal pit **pso** preapical setose organ **sp** spermatheca **spg** spermathecal gland **spgd** spermathecal gland duct **st** trichoid setae. Scale bars: 0.5 mm (**26**, **28**); 0.2 mm (**23–25**); 0.125 mm (**27**).

Abdomen. Ventrites 2–7 with a very fine pubescence. Seventh ventrite with a pair of small ventral setae at its posterior margin. Male genital segment oval, GSL: 0.96 mm, GSW: 0.56 mm.

Aedeagus. Median lobe of aedeagus slightly curved ventrally and elongate, narrowed apically, apical margin rounded (Figs 23–25), MLA: 0.82 mm, OML: 0.42 mm. Left paramere subtriangular, conchoid, about twice as long as wide, LPL: 0.30 mm; right paramere styliform, about three times as long as wide, distinctly shorter than the left one, RPL: 0.16 mm.

Female reproductive tract. Ovipositor (Figs 27, 28): with a broad laterotergite; basal gonocoxite 1 longer than apical gonocoxite 2, with three long trichoid setae apicoventrally, in addition to a smaller trichoid setae observed only on the right gonocoxite 1; gonocoxite 2 strongly curved, falciform in lateral aspect, with slightly rounded apex, with preapical setose organ circuloid ventrally, with four nematiform setae, laterodorsal surface with many marginal pit pegs medially (on the lateroventral surface, the marginal pit pegs are located more apically). Female genital tract totally membranous (Fig. 26). Bursa copulatrix bulbous, expanded in the bursal sacculus anterior to the common oviduct, which is curved to the right basally. Spermatheca, spermathecal gland duct, and spermathecal gland were broken and were not represented or visualized. No secondary spermathecal gland observed.

Distribution. This species is endemic to caves of the region known as “Serra Sul de Carajás” and is currently known to occur in two caves located approximately 10 km from each other (South Mountain, Flona de Carajás, Parauapebas, state of Pará, northern Brazil) (Figs 2, 4, 7).

***Coarazuphium xingu* Pellegrini, Ferreira & Vieira, sp. nov.**

<http://zoobank.org/B336E6F0-8012-47BF-9E43-C1815EE4B0CC>

Figs 29–38

Type material. Holotype: BRAZIL: Pará, São Félix do Xingu, Cave SFX-0057, 6°24'06.2"S, 51°54'08.1"W, ♂, 03.II.2018, Ativo Ambiental leg. (ISLA 75762).

Paratype: the same locality as for holotype, 1 ♀, 20.VII.2018, Ativo Ambiental leg. (ISLA 65430).

Etymology. The epithet *xingu* is given in designation to the type locality, where the two known specimens were collected. *Xingu* is an indigenous word that means “good and clean water” and names one of the main rivers in the region and an important indigenous reserve, the Parque Indigene do Xingu, currently the largest indigenous reserve in Brazil and one of the most important barriers to advanced agricultural development in the Amazon.

Differential diagnosis. All characteristics of *C. xingu* sp. nov. are consistent with the description of the genus *Coarazuphium*. This species differs from all others of the genus by the following combination of characters: elytral outline subparallel, elytra with maximum width in the posterior half, with a very slight subapical sinuosity; loca-

tion of setigerous punctures on the head dorsally: one anterior supraorbital and one postocular; antennae not very long, about 0.68 times as long as body length; metafemur without a spine medially at the ventral side.

Description. Size and proportions. OBL: 3.26 mm ♂, 3.19 mm ♀, EW: 1.09 mm ♂, 1.10 mm ♀, HW/PW: 1.07 ♂, 1.10 ♀.

Habitus. Body with uniform pale to dark brown color (Fig. 30).

Integument. Dorsally covered with short recumbent hairs.

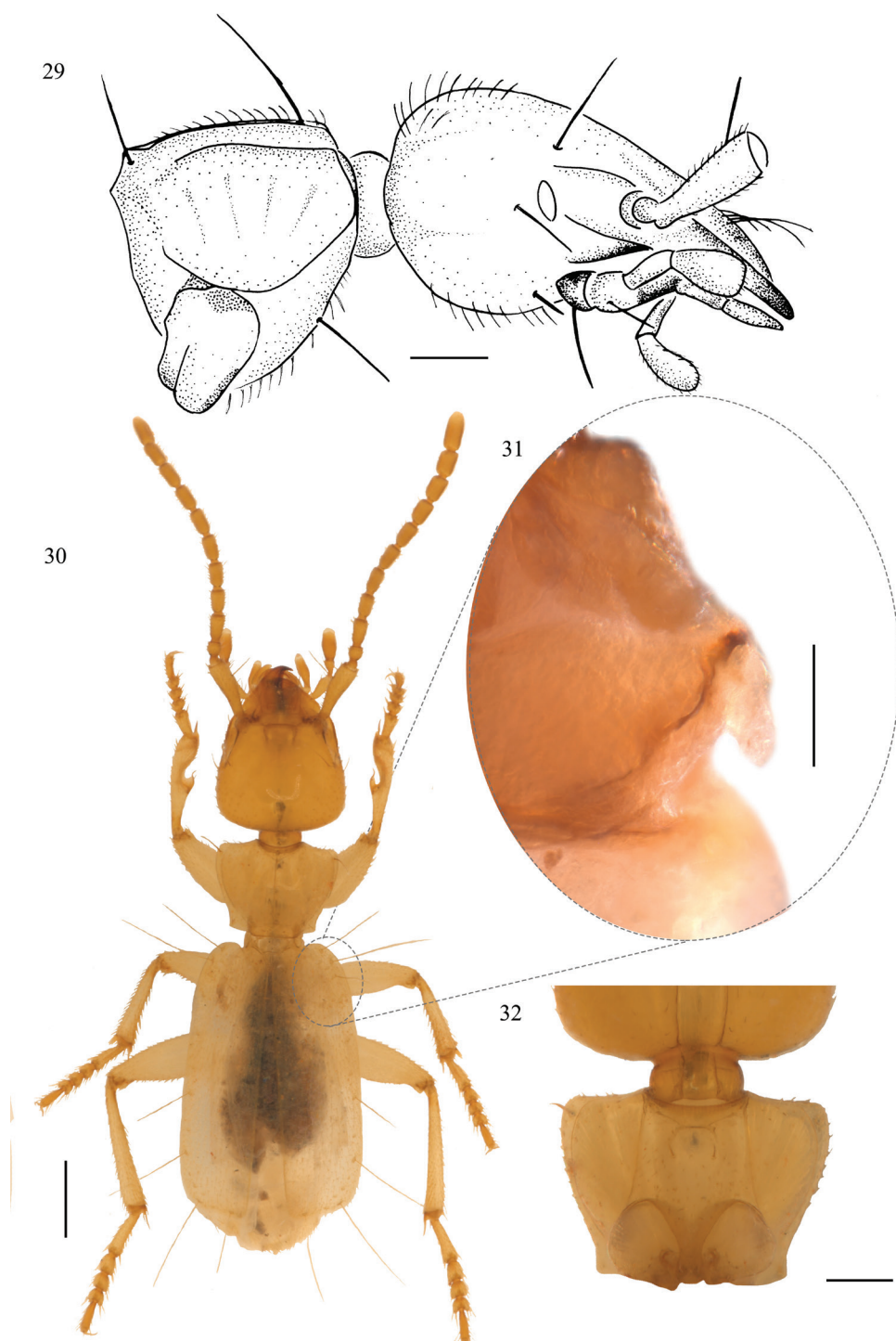
Head. Subtrapezoidal (Fig. 30), HW/HL: 0.91 ♂, 0.96 ♀. Head almost as wide as pronotum. Location of setigerous punctures on the head dorsally: one pair of anterior supraorbital above the eyes and one pair of postocular; head is covered with a fine pubescence more densely distributed on the vertex margin; ventrally are three pairs of setae on the post-gena located apically and a fine pubescence on the gula (Fig. 29). Eyes reduced, depigmented, and flattened, situated laterally at the end of the genal sulcus, ommatidia are not visible at 50× (Fig. 29). Antennae filiform and flagellar (Fig. 30), AL: 2.21 mm ♂, 2.11 mm ♀, AL/PL: 3.74 ♂, 3.67 ♀, A1L/A2–4L: 0.76 ♂, 0.75 ♀. First antennomere (scape) with a long seta distally close to the apical portion, and a row of several semi-erect setae; 2nd one very short. Antennal segments 3–10 subequal, rectangular, and almost round in cross-section, except for the tip of the terminal antennomere, which is laterally flattened.

Prothorax. Pronotum trapezoidal, PL/PW: 0.75 ♂, 0.79 ♀ (Figs 29, 30). Maximum pronotum width closely behind the anterior margin, which is almost as wide as head. Anterior angle rounded. Posterior angle acute. Dorsal surface with two pairs of lateral marginal erect setae: one close to the antero-lateral angles, and the other shorter, close to the postero-lateral angles. Prosternum with a pair of sub-medial setae (Fig. 32).

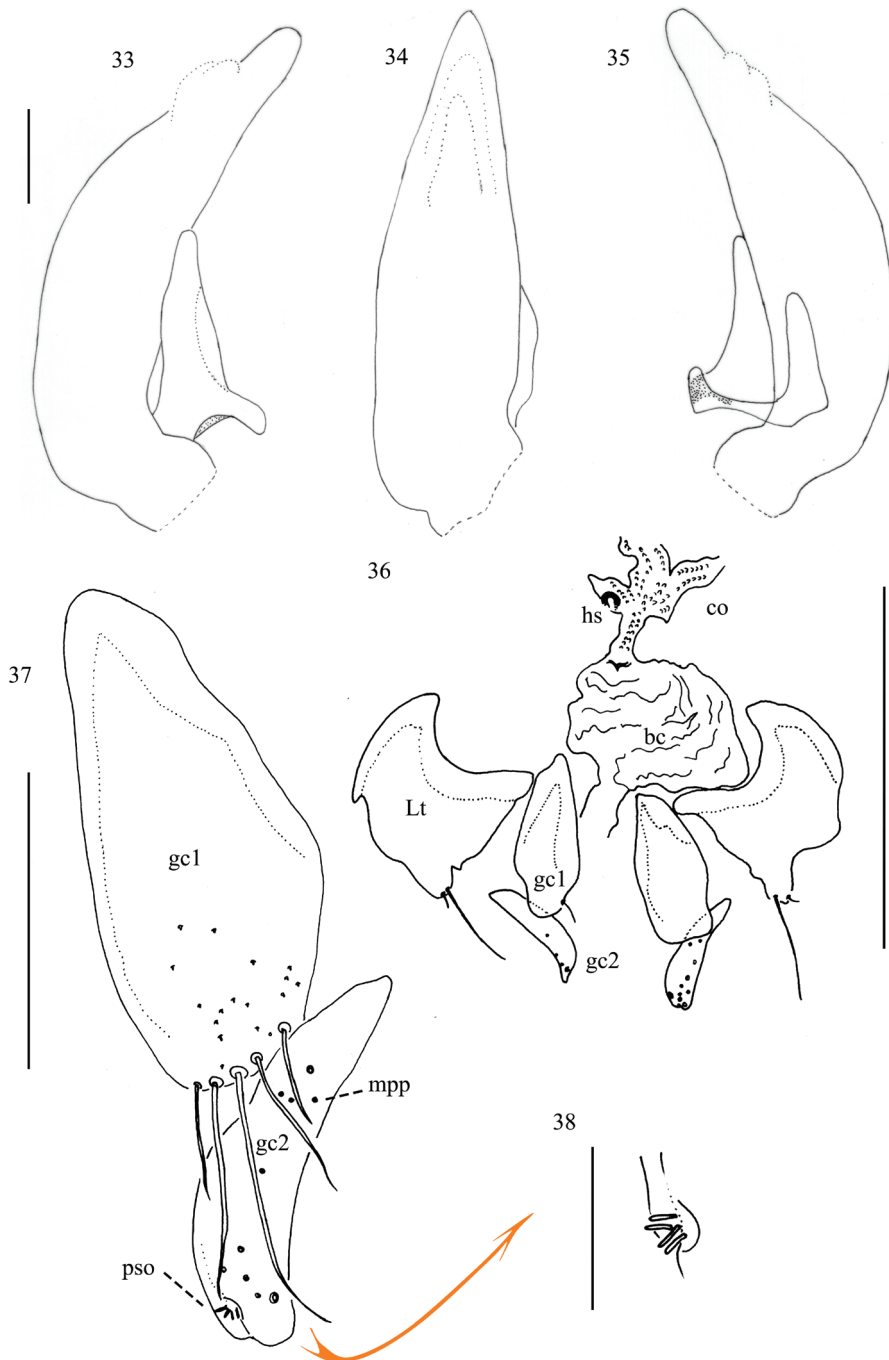
Pterothorax. Metasternum longer than wide. Metepisternum wider than long.

Elytra and hind wings. Elytra free (Fig. 30), EL/EW: 1.60 ♂, 1.62 ♀. Elytral outline subparallel, maximum elytral width in the posterior half, EW/PW: 1.39 ♂, 1.50 ♀. Subapical elytral sinuosity very slight. Elytral chaetotaxy: no discal setae present; the umbilicate series of the 8th stria with seven large setae (about 0.58 times as long as elytra) on each elytron: three close to the anterior angle, two marginal in the lateral posterior half, and two on the posterior margin. Hind wings very reduced (Fig. 31), 0.10-mm long, HWL/EL: 0.06 ♂.

Legs. Profemur 1.21 (♂) and 1.12 (♀) times as long as mesofemur, and 0.84 (♂) and 0.76 (♀) times as long as metafemur, respectively. Protibia 1.23 (♂) and 1.05 (♀) times as long as mesotibia, and 0.79 (♂) and 0.75 (♀) times as long as metatibia, respectively. Protibia 1.28 (♂) and 1.20 (♀) times as long as protarsus, mesotibia 0.81 (♂) and 0.97 (♀) times as long as mesotarsus, and metatibia 0.93 (♂) and 1.01 (♀) times as long as metatarsus, respectively. First pro-, meso-, and metatarsomere each almost equal to tarsomeres 2–4 combined. Length of protibia and protarsus combined 2.04 (♂) and 2.01 (♀) times as long as pronotum, length of mesotibia and mesotarsus 2.09 (♂) and 2.11 (♀) times as long as pronotum, while length of metatibia and metatarsus 2.99 (♂) and 2.91 (♀) times as long as pronotum, respectively.



Figures 29–32. *Coarazuphium xingu* sp. nov., external morphology **29** head and prothorax, lateral view **30** habitus, dorsal view **31** a detail on hind wings, dorsal view **32** prothorax, ventral view. Scale bars: 0.5 mm (**30**), 0.2 mm (**29**, **32**); 0.1 mm (**31**).



Figures 33–38. *Coarazuphium xingu* sp. nov., male and female genitalia **33** aedeagus, left lateral view **34** aedeagus, dorsal view **35** aedeagus, right lateral view **36** female reproductive tract, dorsal view **37** gonocoxite, dorsal view **38** preapical setose organ, dorsal view **bc** bursa copulatrix **co** common oviduct **gc1** gonocoxite 1 **gc2** gonocoxite 2 **hs** helminthoid sclerite **Lt** laterotergite **mpp** marginal pit pegs **pso** preapical setose organ. Scale bars: 0.5 mm (**36, 38**); 0.125 mm (**37**); 0.1 mm (**33–35**).

Abdomen. Ventrites 2–7 with a very fine pubescence. Seventh ventrite with a pair of small ventral setae at its posterior margin. Male genital segment triangular, GSL: 0.65 mm, GSW: 0.38 mm.

Aedeagus. Median lobe of aedeagus slightly curved ventrally and elongate, narrowed apically, apical margin rounded (Figs 33–35), MLA: 0.58 mm, OML: 0.15 mm. Left paramere subtriangular, conchoid, about twice as long as wide, LPL: 0.21 mm; right paramere styliform, about three times as long as wide, distinctly shorter than the left one, RPL: 0.15 mm.

Female reproductive tract. Ovipositor (Figs 37, 38): with a broad laterotergite; basal gonocoxite 1 longer than apical gonocoxite 2, with two small and three long trichoid setae apicoventrally; gonocoxite 2 strongly curved, falciform in lateral aspect, with notched apex, with preapical setose organ circuloid ventrally, with four nematiform setae, laterodorsal surface with many marginal pit pegs medially (on the lateroventral surface, the marginal pit pegs are located more apically). Female genital tract totally membranous (Fig. 36). Bursa copulatrix bulbous, expanded in the bursal sacculus anteriorly to the insertion point of common oviduct, which is curved basally and partially broken. Spermatheca, spermathecal gland duct, and spermathecal gland were broken and were not represented or visualized. No secondary spermathecal gland observed.

Distribution. The species was found in a single cave located in the municipality of São Felix do Xingu, state of Pará, Brazil (Figs 2, 4, 5).

Comparative diagnosis

The *Coarazuphium* species that occur in the Carajás region are morphologically similar, those are likely each other's closest living relatives, and are distributed in a small geographic range. From the six known species, two stand out: *C. spinifemur* and *C. xingu* sp. nov., which are the smallest *Coarazuphium* species. They both possess shorter legs and antennae when compared to the remaining species. The main characteristic that distinguishes *C. xingu* sp. nov. from *C. spinifemur* is the presence of a femoral spine in the latter species. In addition to the general proportions of the body (i.e., less elongated antennae and legs), other combination of characters – such as the existence of only two dorsal setigerous punctures on the head, head quadrangular and elytra with almost parallel sides, with the apical margin only slightly sinuated – also makes *C. xingu* sp. nov. unique morphologically.

With an overall body form very similar to that of the other two species from the Carajás region (*C. tapiaguassu* and *C. amazonicum*), the main characteristic that distinguishes *C. xikrin* sp. nov. and *C. kayapo* sp. nov. from the above mentioned two species is the number of setigerous punctures dorsally on the head. The two former species have only two pairs of fixed setae dorsally on the head: one pair of anterior supraorbital and one pair of postocular. Besides those setae, *C. xikrin* sp. nov. also bears one pair of posterior supraorbital setae. *Coarazuphium kayapo* sp. nov. possesses, in addition to the three pairs of setae mentioned, one pair of posterior supernumerary setae and two pairs of occipital setae. *Coarazuphium xikrin* sp. nov. can be distinguished from all other *Coarazuphium* species by possessing the described

setae posteriorly on the head, quadrangular head, elytra with almost parallel sides, with the apical margin truncate. *Coarazuphium kayapo* sp. nov. can be distinguished from the other *Coarazuphium* species by possessing the described setae posteriorly on the head, quadrangular head, elytra with almost parallel sides, with the apical margin only slightly sinuated.

Key for identification of adults of species of the genus *Coarazuphium* (modified after Pellegrini et al. 2021)

- 1 Elytra with apical margin truncate, not sinuate (Pellegrini and Ferreira 2011: 49, Fig. 2A) **2**
- Elytra with apical margin sinuate (Godoy and Vanin 1990: 796, Fig. 1) or with a slight apical sinuosity (Pellegrini et al. 2020: 291, Fig. 5) **9**
- 2(1) Head dorsally bearing two pairs of setae: one pair of anterior supraorbital and one pair of postocular (Pellegrini and Ferreira 2017: 556, Fig. 5) **3**
- Head dorsally bearing three or more pairs of setae, rarely two pairs. If only two setae are present, the postocular seta is absent **5**
- 3(2) Metafemur with a spine medially at the ventral side (Pellegrini and Ferreira 2017: 556, Fig. 4), antennae short, about 0.68 times as long as total body length ***C. spinifemur* Pellegrini & Ferreira, 2017**
- Metafemur without a spine medially at the ventral side, antennae long, reaching metafemur insertion **4**
- 4(3') Aedeagus very long and slender, about 2.89 times as long as left paramere (Pellegrini and Ferreira 2011: 49, Figs 2D–F) ***C. tapiaguassu* Pellegrini & Ferreira, 2011**
- Aedeagus shorter, about 2.60 times as long as left paramere (Pellegrini and Ferreira 2017: 557, Figs 6C–E) ***C. amazonicum* Pellegrini & Ferreira, 2017**
- 5(2') Head dorsally bearing only two pairs of setae: one pair of anterior supraorbital and one pair of posterior supraorbital (Bená and Vanin 2014: 291, Fig. 5) ... ***C. ricardoi* Bená & Vanin, 2014**
- Head dorsally with three or more pairs of setae: at least one pair of anterior supraorbital, one pair of postocular and one pair of posterior supraorbital .. **6**
- 6 Head dorsally with three pairs of setae (Pellegrini et al. 2021: 6, Fig. 8) ***C. auleri* Pellegrini & Vieira, 2021**
- Head dorsally with more than three pairs of setae **7**
- 7(6') Five pairs of setae on the head dorsally: occipital and posterior supraorbital setae placed beyond anterior supraorbital, postocular and posterior supernumerary setae (Ball and Shpeley 2013: 30, Fig. 4A) ***C. whiteheadi* Ball & Shpeley, 2013**
- Four pairs of setae on the head dorsally **8**
- 8(7') Besides anterior supraorbital, postocular and posterior supernumerary setae, head dorsally bearing one pair of posterior supraorbital setae, located more anterior and lateral than posterior supernumerary setae (Pellegrini et al. 2020: 297, Fig. 22) ***C. pains* Álvares & Ferreira, 2002**

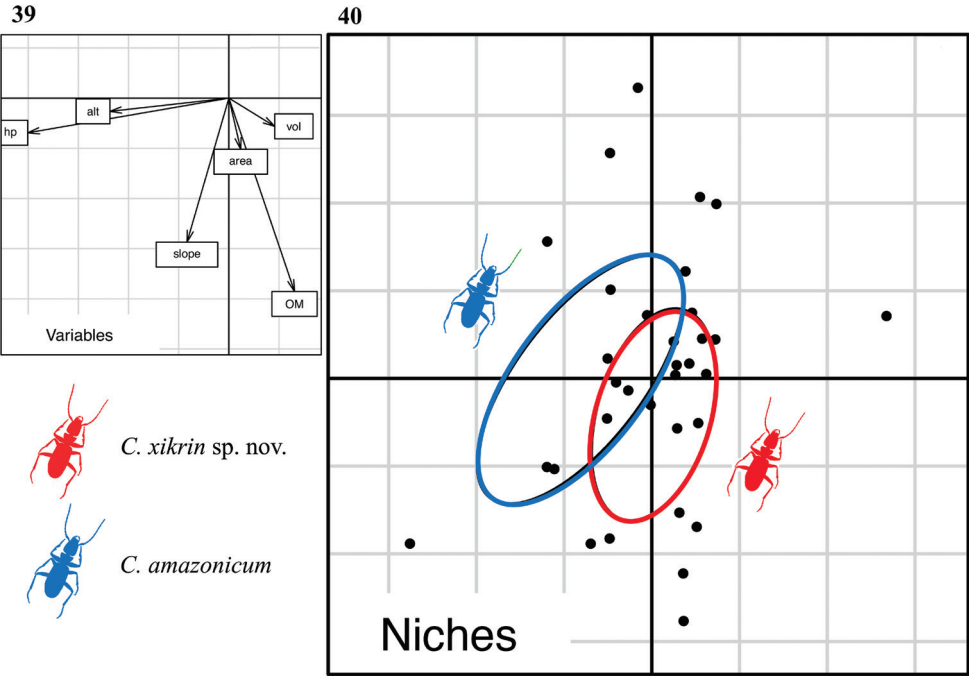
- Besides anterior supraorbital, postocular and posterior supernumerary setae, head dorsally bearing one pair of occipital setae, located more posterior and medially than posterior supernumerary setae (Fig. 8) ***C. xikrin* sp. nov.**
- 9(1') Head dorsally bearing two to three pairs of setae, anterior supraorbital and postocular setae always present **10**
- Head dorsally bearing four to five pairs of setae, anterior supraorbital, postocular, posterior supernumerary and occipital setae always present **13**
- 10(9') Head dorsally bearing only two pairs of setae: one pair of anterior supraorbital and one pair of postocular (Fig. 29) ***C. xingu* sp. nov.**
- Head dorsally bearing three pairs of setae **11**
- 11(10) Head elongate, HW/HL about 0.60
..... ***C. cessaima* Gnaspini, Vanin & Godoy, 1998**
- Head subquadrangular, HW/HL 0.95 **12**
- 12(11') Left paramere styliform (Godoy and Vanin 1990: 798, Fig. 2), elytra with one pair of short setae behind scutellum and two pairs of discal setae (Godoy and Vanin 1990: 798, Fig. 1) ***C. tessai* (Godoy & Vanin, 1990)**
- Left paramere broad, conchoid (Pellegrini et al. 2022: 564, Fig. 11), elytra without discal setae or any setae behind scutellum
..... ***C. bambui* Pellegrini & Vieira, 2022**
- 13(9') Head dorsally with two pairs of occipital setae (Pellegrini and Ferreira 2014: 529, Fig. 2B) **14**
- Head dorsally with one pair of occipital setae **16**
- 14(13) Head dorsally having a pubescence (Fig. 18), with posterior medium-sized setae anterior and lateral to posterior supernumerary setae ***C. kayapo* sp. nov.**
- Head dorsally glabrous, except for fixed setae **15**
- 15(14) Elytra with a slight apical sinuosity (Pellegrini and Ferreira 2014: 537, Figs 12A, C), head dorsally without protuberances
..... ***C. formoso* Pellegrini & Ferreira, 2011**
- Elytra with a pronounced apical sinuosity, head dorsally with two protuberances (Pellegrini and Ferreira 2014: 537, Figs 12B, D)
..... ***C. caatinga* Pellegrini & Ferreira, 2014**
- 16(13') Head dorsally bearing one pair of posterior supernumerary setae (Pellegrini et al. 2020: 293, Fig. 6) ***C. lundi* Pellegrini, Ferreira & Vieira, 2020**
- Head dorsally without posterior supernumerary setae
..... ***C. bezerra* Gnaspini, Vanin & Godoy, 1998**

Habitat preferences and niche overlap

Comparing niches from two *Coarazuphium* species, *C. amazonicum* and *C. xikrin* sp. nov., revealed that the latter species is more tolerant to the average environmental conditions available in the caves located at Serra Norte de Carajás (Table 1, Figs 39, 40). *Coarazuphium amazonicum*, in turn, is preferentially associated to caves with wider horizontal projection.

Table I. OMI analysis results.

	Inertia	OMI	Tol	Rtol	p-value
<i>C. amazonicum</i>	6.257	0.662	1.422	4.174	0.05
<i>C. xikrin</i> sp. nov.	4.974	0.176	1.442	3.356	0.05
OMI mean					0.05



Figures 39–40. Results of the outlying mean index (OMI) for *C. xikrin* sp. nov. and *C. amazonicum* **39** principal component analysis (PCA) of the environmental variables showing the covariation of variables in relation to the cave and the niche representation **40** representation of the occupied niche for the analyzed species in relation to the environmental variables. The black dots represent the sampling points, corresponding to each cave **alt** altitude **area** the area of the cave floor **hp** horizontal projection **OM** diversity of organic matter resources available **slope** the slope of the cave floor **vol** cave volume

Discussion

Niche overlap patterns of two co-existing species

Ecological theory predicts that high competition rates reduce a species' realized niche, and this competition is often intensified in cave environments (Culver 1970). The presence of related species in an environment with low-resource availability, such as caves, intensifies intra- and interspecific competition pressure, especially among top predator species (Mammola et al. 2016). On small scales, co-existing species tend to evolve divergent habitat preferences that allow their coexistence in the cave's harsh habitat (Mammola et al. 2016; Martins and Ferreira 2020).

We found evidence for niche differentiation between *C. xikrin* sp. nov. and *C. amazonicum*. *Coarazuphium xikrin* sp. nov. is apparently more tolerant to the average conditions available in the caves of the Carajás region, while *C. amazonicum* is preferentially associated with larger caves. It is well known by the speleological community that bigger caves shelter a higher number of species. The species-area relationship has been empirically tested and confirmed in a variety of taxonomic groups (Schneider and Culver 2004; Souza-Silva and Ferreira 2011; Ferreira and Pellegrini 2019; Souza-Silva et al. 2020), and larger caves are generally associated with higher availability of food resources and with higher environmental heterogeneity. Hence, bigger caves typically possess more niches available for colonization.

A hotspot for troglobitic beetles in South America

Considering the current distribution of *Coarazuphium* species, the iron ore caves of the southeastern portion of the state of Pará, especially the Carajás region, shelter the highest known diversity of the genus. So far, six *Coarazuphium* species have been described from this region, and this richness corresponds to approximately 40% of the known species. Most *Coarazuphium* species, especially those associated with limestone caves, are infrequently collected and endemic (for several species only a few specimens are known). However, in the Carajás region, certain species are abundant and distributed in dozens of caves, including cases of sympatry, as observed for *C. amazonicum* and *C. xikrin* sp. nov. in the Serra Norte area, and for *C. tapiaguassu* and *C. spinifemur* in the Serra Leste area (Fig. 4).

The Carajás region corresponds to one of the most important mineral reserves in Brazil, and the current protection of the caves by the Brazilian government represents a major environmental obstacle to overexploitation of such mineral resources. Therefore, the maintenance of legal provisions that ensure the preservation of caves is crucial for the conservation of Brazilian subterranean biodiversity. This is even more important for the most relevant caves regarding physical and biotic aspects, especially in this region that represents a hotspot of cave beetles. In addition to the six *Coarazuphium* species, several other troglobitic beetle taxa are known to occur in caves in the region, including the following: *Copelatus cessaima* Caetano, Bená & Vanin, 2013 (Dytiscidae), *Ardistomis ferreirai* Balkenohl, Pellegrini & Zampaulo, 2018 (Clivinini, Carabidae), and *Metopioxys carajas* Asenjo, 2019 (Pselaphinae, Staphylinidae). Furthermore, several other troglobitic species from different invertebrate groups (mostly arthropods) also exist in Carajás, including spiders – Oonopidae (many species), Caponiidae (*Carajas paraua* Brescovit & Sánchez-Ruiz, 2016), Gnaphosidae (*Pracymbionna carajas* Rodrigues, Cizauskas & Rheims, 2018), Tetrablemmidae (*Matta* sp.), and Ochyroceratidae (*Speocera* spp., *Ochyrocera* spp.); tailless whip scorpions – Charinidae (*Charinus ferreus* Giupponi & Miranda, 2016); harvestmen – Escadabiidae (many species); pseudoscorpions – Bochicidae, Chthoniidae, and Ideoroncidae (many species); millipedes – Glomeridesmidae (*Glomeridesmus spelaus* Iniesta, Ferreira & Wesener, 2012), Pyrgodesmidae (many species), and Pseudonannolenidae (*Pseudonannolene* spp.); springtails – Paronellidae (*Trogolaphysa* sp., *Cyphoderus* sp.), Entomobryidae (*Pseudosinella* sp.), and Sminthuridae

(*Pararrhopalites* sp.); woodlice – Scleropactidae (*Circoniscus* spp.) and Calabozoidae; amphipods – Bogidiellidae (*Bogidiella* sp.); and flatworms – Prorhynchidae (*Geocentrophora* sp.) (Brescovit et al. 2021). Bento et al. (2021) discussed about the Brazilian areas of great biospeleological relevance. Amongst the 10 regions mentioned in this work, the Carajás region stood out as the second in absolute number of troglobitic species. The area with the highest number of cave-restricted species comprised the Atlantic Forest as a whole, and this high number is certainly a result of the large extension of this domain, in comparison with the other more spatially restricted areas presented. Furthermore, it should be noted that the Carajás region also possesses a very unique troglobitic fauna, with species belonging to groups that only occur in this region (Ferreira et al. 2018).

The Xingu River basin region represents a large corridor of protected areas and its protection remains one of the most effective barriers against deforestation in the Brazilian Amazon. However, studies carried out by non-governmental associations reveal a significant increase in deforestation in the region in the last few years. Between 2018 and 2020, 513,500 hectares were deforested in the Xingu basin. Instead of implementing measures to protect the Xingu Reserve, the Brazilian government has promoted a scenario of total impunity through rhetoric favorable to the unconstitutional reduction of indigenous lands and the legalization of destructive activities, such as mining, in addition to weakening oversight (Instituto Socioambiental – ISA 2020; MapBiomas 2021). Therefore, despite the high biological relevance of those areas, which shelter an exceptionally high set of unique subterranean fauna, all the cave-restricted species are more threatened than ever.

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Two new troglobitic species of *Iansaoniscus* from Brazilian caves (Crustacea, Isopoda, Pudeoniscidae)

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Abstract

Iansaoniscus species are troglobitic terrestrial isopods of the Pudeoniscidae family. In this work two new species are described from caves in the Bahia state, northeastern Brazil; *I. leilae* **sp. nov.** from Toca do Gonçalves cave, in the municipality of Campo Formoso; and *I. paulae* **sp. nov.** from Lapa do Bode cave in the municipality of Itaeté. Additionally, ecological notes and conservation status are provided for both new species.

Keywords

Cave fauna, Neotropics, Oniscidea, terrestrial isopods, troglobites

Introduction

Brazil currently shelters 36 described species of troglobitic oniscidean isopods, and this number has highly enhanced in the last few years (Cardoso et al. 2021, 2022; Campos-Filho et al. 2022). New species have continuously been discovered as new karstic areas are sampled. Styloniscidae stands out among the families with cave-restricted taxa, with 25 species (Fernandes et al. 2019; Cardoso et al. 2021, 2022; Campos-Filho et al. 2022); followed by Scleropactidae with five species (Campos-Filho and Araujo 2011; Campos-Filho et al. 2014, 2020), Philosciidae with four species (Campos-Filho et al. 2019, 2020), and Pudeoniscidae with two species (Campos-Filho et al. 2017).

Pudeoniscidae present nine species in four genera: *Brasiloniscus* Lemos de Castro, 1973, *Pudeoniscus* Vandel, 1963, *Iansaoniscus* Campos-Filho, Araujo & Taiti, 2017, and *Oxossioniscus* Campos-Filho, Lisboa & Cardoso, 2018 (Vandel 1963; Lemos de Castro 1973; Campos-Filho et al. 2017, 2018). Most species of this family occur in the Brazilian Atlantic Forest, with representatives found associated with leaf litter of cocoa, bromeliads, canopy of *Erythrina fusca* Lour., termite nests, and caves (Campos-Filho et al. 2017, 2018).

To date, *Iansaoniscus* is the only genus of this family with troglobitic species (Campos-Filho et al. 2017). The genus comprises two species: *Iansaoniscus georginae* Campos-Filho, Araujo & Taiti, 2017 from Borboletas cave, municipality of Paripiranga and *Iansaoniscus iraquara* Campos-Filho, Araujo & Taiti, 2017 from Buraco do Cão cave, municipality of Iraquara, both in Bahia state.

The genus is distinguished by the unique shape of cephalon with well-developed antennary lobes and the triangular frontal shield, that is laterally interrupted by antennal grooves; pereonite 1 epimeron with dorsolateral furrow reduced or absent; pleon outline continuous with pereon; telson triangular; uropod protopod subquadangular; and pleopod exopods with no respiratory structures (Campos-Filho et al. 2017).

In the present work, two new troglobitic species of *Iansaoniscus* are described, both from limestone caves in the Bahia state. Furthermore, ecological notes and conservation status are provided for both new species.

Materials and methods

The new species were manually collected, fixed in 70% ethanol, and taken to the Center of Studies on Subterranean Biology of the Federal University of Lavras (CEBS, UFLA), Lavras, Brazil. Photographs and measurements were taken with ZEISS Axio ZoomV16 stereomicroscope coupled with an Axio Cam 506 Color camera. The specimens were dissected and mounted with Hoyer's medium (Anderson 1954). Illustrations were based on photographs or taken with the aid of a camera lucida on a microscope Leica DM750, and digitally drawn using the software GIMP (v. 2.8) (Montesanto 2015, 2016). Some specimens were dried in alcoholic series for the analysis of dorsal cuticular structures using the scanning electron microscope Hitachi TM4000. The material is deposited in the Collection of Subterranean Invertebrates of Lavras (ISLA), UFLA.

Taxonomy

Family Pudeoniscidae Lemos de Castro, 1973

Genus *Iansaoniscus* Campos-Filho, Araujo & Taiti, 2017

Type species. *Iansaoniscus iraquara* Campos-Filho, Araujo & Taiti, 2017, by original designation.

***Iansaoniscus leilae* sp. nov.**

<http://zoobank.org/4F20CBD9-585B-48BF-9C18-019EFF7B5EEF>

Figs 1–4

Scleropactidae sp. nov. [sic!] – Souza-Silva and Ferreira 2016: 9, fig. 3F.

Diagnosis. Cephalon lateral lobes with straight distal margin; frontal shield triangular with frontal margin directed upwards with antennal socket depressed, vertex margin depressed; pereonite 1 epimeron without dorsolateral furrow; pereonite 1 grooved on ventral margin for two thirds of its length, pereonite 2 with well-developed ventral lobe; telson with acute apex; antennula distal article with two lateral and two apical aesthetascs; uropod exopod slightly shorter than endopod, as long as distal margin of telson.

Material examined. Holotype: BRAZIL • 1 male; Bahia state, municipality of Campo Formoso, Toca do Gonçalves cave (WGS84 -10.510992, -40.894659); 10th December 2018; RL Ferreira leg.; ISLA 95911. **Paratypes:** • 1 male (mounted in slide), 2 females (1 mounted in slide); same data as holotype; ISLA 95912. • 1 male, 1 female; 05th January 2008; same location as holotype; ISLA 95913. • 6 females; 05th January 2008; same location as holotype; ISLA 95914. • 1 male (mounted in slide), 1 female; municipality of Campo Formoso, Toca da Tiquara cave (-10.458611, -40.542222); RL Ferreira leg.; ISLA 95917.

Etymology. The new species is named after Dr. Leila Aparecida Souza, UECE, for her contributions on the systematics of Brazilian subterranean Oniscidea.

Description. Maximum length: male and female, 6 mm. Body outline in lateral view as in Fig. 1A, colorless, eyes absent (Fig. 1A–C). Cephalon lateral lobes with straight distal margin; frontal shield triangular with frontal margin directed upward with antennal socket depressed, vertex margin depressed (Fig. 1B, C). Pereonite 1 epimeron without dorsolateral furrow; pereonite 1 grooved on ventral margin for two thirds of its length, pereonite 2 with well-developed ventral lobe (Fig. 1D–F); pereonites 3–7 with sub-quadrangular epimera. Pleonites 3–5 with epimera sub-quadrangular and directed backwards (Figs 1A, G, 2A). Telson (Figs 1G, 2A) slightly wider than long with concave sides, apex acute. Antennula (Fig. 2B) with three articles, distal article longer than second article bearing two lateral and two apical aesthetascs. Antenna (Fig. 2C) surpassing pereonite 3 when extended backwards, fifth article of peduncle as long as flagellum; flagellum with three articles; second and third articles subequal in length, third article with lateral aesthetascs (Fig. 1H). Mandibles (Fig. 2D, E) molar penicil with 7–8 branches, left mandible with 2+1 penicils, right with 1+1 penicils. Maxillula (Fig. 2F) outer branch with 4 + 5 teeth (two apically cleft); inner branch with two penicils. Maxilla (Fig. 2G) with bilobate apex, outer lobe wider than inner lobe, round, covered with thin setae; inner lobe bearing thick setae. Maxilliped (Fig. 2H) basis rectangular; palp basal article with two setae distinct in length; endite sub-rectangular, long medial seta, distal margin with two teeth. Pereopods 1–7 (Fig. 3A, B) merus and carpus with sparse setae on sternal margin; carpus 1 with longitudinal antennal grooming brush, distal seta apically cleft; dactylus with dactylar organ and

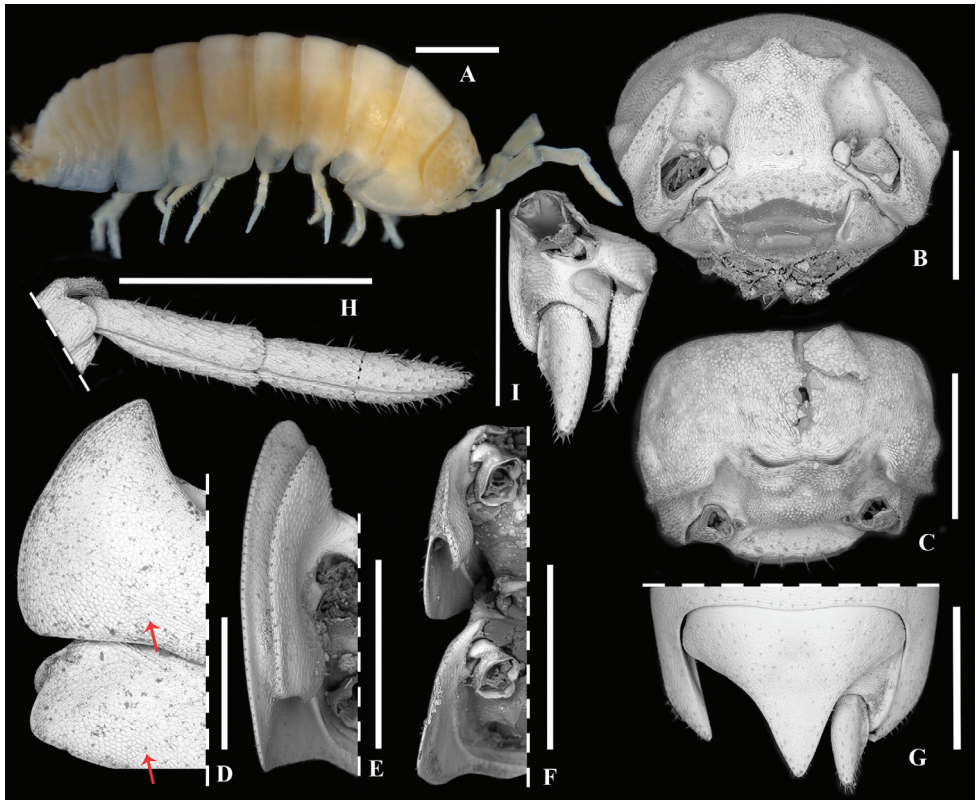


Figure 1. *Iansaoniscus leilae* sp. nov. **A** male holotype, lateral view. Female paratype **B** cephalon, frontal view **C** cephalon, dorsal view **D** pereonites 1 and 2, dorsal view, arrow indicating the *noduli laterales* **E** pereonite 1, ventral view **F** pereonites 2 and 3, ventral view **G** pleonite 5, telson and uropod, dorsal view **H** antennal flagellum **I** uropod. Scale bars: 1 mm (**A**); 500 µm (**B**–**I**).

ungual setae simple not surpassing outer claw. Uropod (Figs 1G, 2A) protopod longer than wide, with lateral groove; endopod inserted proximally, exopod slightly shorter than endopod, exopod slightly longer than distal margin of telson; proximal margin with lateral groove

Male: Genital papilla (Fig. 3C) with triangular ventral shield and subapical orifices. Pleopod 1 (Fig. 3C) exopod ovoid, margin straight, almost three times wider than long; endopod four times as long as exopod, distal portion slightly bent outwards, apex with small setae. Pleopod 2 exopod (Fig. 3D) triangular bearing one seta on outer margin, endopod flagelliform, almost three times as long as exopod. Pleopod 3–5 exopods as in Fig. 3E–G.

Habitat and conservation issues. The Toca do Gonçalo cave presents ca. 500 meters of mapped conduits. It is inserted at the Una geological group of carbonate rocks from the Caatinga Formation, which comprises relatively young rocks deposited around 600 million years ago (Auler 2019). It is located in the Campo Formoso

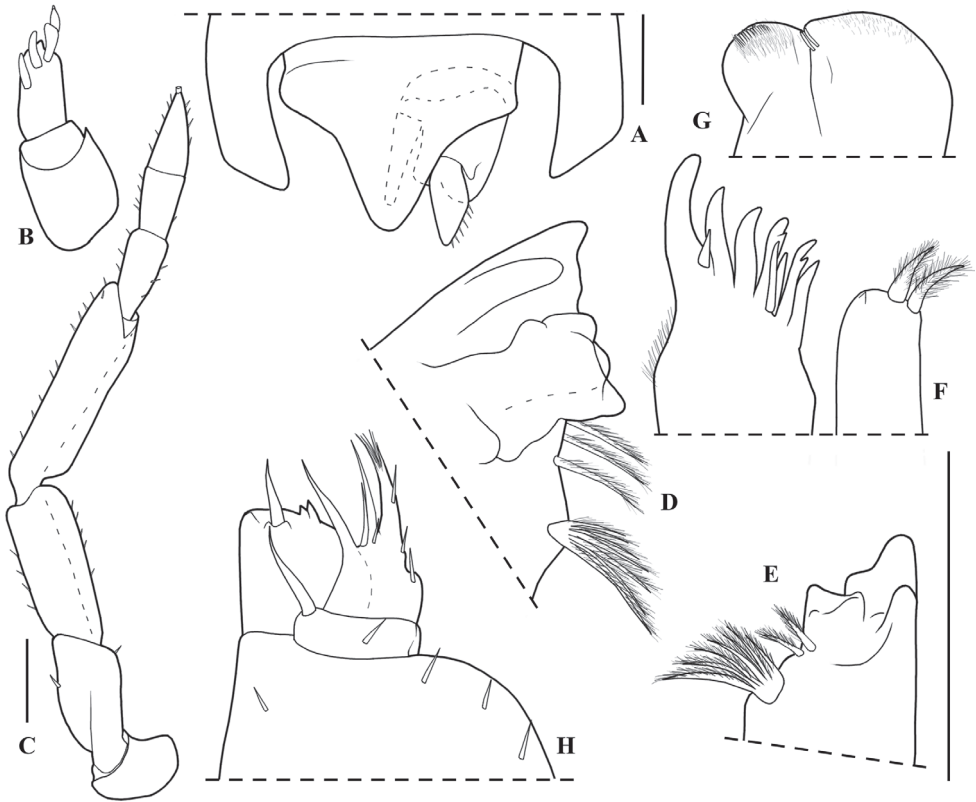


Figure 2. *Iansaoniscus leilae* sp. nov. Male holotype **A** telson and uropod, dorsal view **B** antennula **C** antenna **D** left mandible **E** right mandible **F** maxillula **G** maxilla **H** maxilliped. Scale bars: 0.2 mm.

municipality, Bahia state, Northeastern Brazil, within the Caatinga (a semiarid biome) (Fig. 9). The cave presents a single horizontal lenticular-shaped entrance, 2 m high and 8 m wide (Fig. 4A). It has two distinct levels: while the upper level is predominantly dry, the lower level used to be frequently filled with phreatic water (Fig. 4B, C). However, as will be discussed further on, several changes (at both local and regional scales) led to a massive reduction in the water table level, thus, exposing several conduits that used to be inaccessible to men in the past decades (Fig. 4C). The main food sources for both the terrestrial and aquatic fauna seem to be particulate organic matter from the surface, although there are some root mats in the water table level and small guano piles in some areas within the cave.

Culver and Sket (2000) defined hotspots of subterranean biodiversity as caves (or cave systems) with 20 or more cave-restricted species. The Toca do Gonçalves cave shelters 22 troglobitic species and represents one of the three hotspots of subterranean biodiversity known for South America (Souza-Silva and Ferreira 2016; Souza-Silva et al. 2021). Most troglobitic species occurring in this cave remain undescribed, with descriptions already known for only seven of them: the beetle *Coarazuphium caatinga*

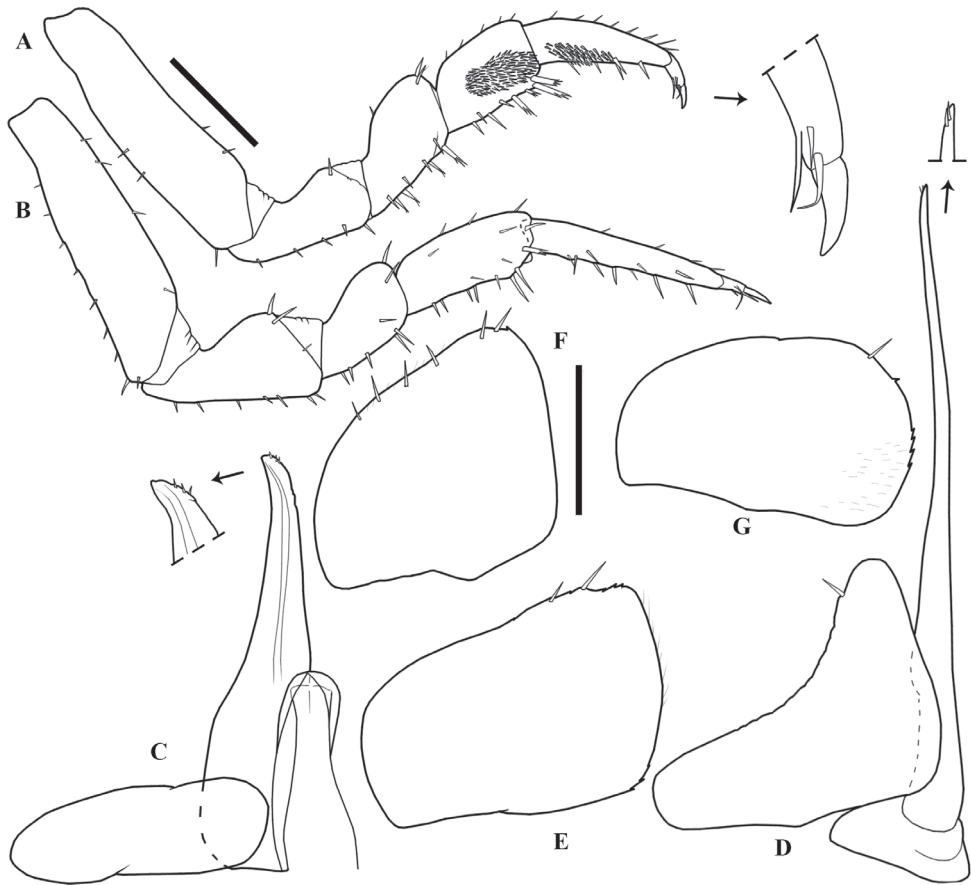


Figure 3. *Iansaoniscus leilae* sp. nov. Male holotype **A** pereopod 1 **B** pereopod 7 **C** pleopod 1 **D** pleopod 2 **E** pleopod 3 exopod **F** pleopod 4 exopod **G** pleopod 5 exopod. Scale bars: 0.2 mm.

(Pellegrini & Ferreira, 2014); the cricket *Erebonyx catacumbae* (Mello & Ferreira, 2021); the paligrade *Allokoenenia canhembora* (Souza & Ferreira, 2022); the centipedes *Cryptops spelaeoraptor* (Ázara & Ferreira, 2014a) and *Newportia spelaea* (Ázara & Ferreira, 2014b); the amphipod *Spelaeogammarus trajanoae* (Koenemann & Holsinger, 2000); and the isopod *Pongycarcinia xyphidiorus* (Messana et al. 2002).

Specimens of *I. leilae* sp. nov. (Fig. 4D, E) were observed at the upper level in the first cave surveys, in the late 1990s. Several specimens were observed, especially near some old bat guano piles. At that moment, most of the lower level was filled with water. Hence, even the upper level was moist due to the influence of the high phreatic level on the whole cave atmosphere. However, local inhabitants use to draw water from the cave (as the region is quite dry) and this practice occurred for decades. This removal occurred manually at the beginning, but a diesel pump (Fig. 4F) was installed inside the cave in the 1980s, drying out previously flooded areas (Prevorčnik et al. 2012; Souza-Silva and Ferreira 2016). It is important to mention that the locals

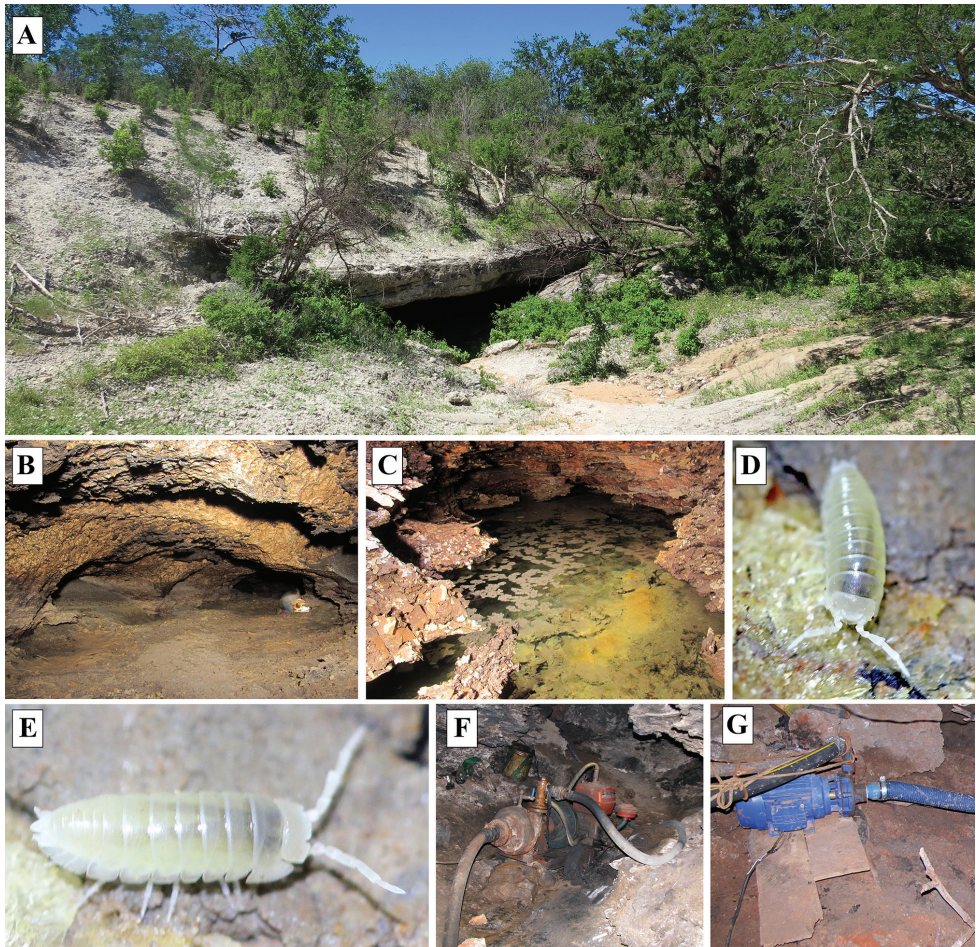


Figure 4. *Iansaoniscus leilae* sp. nov. habitat **A** Toca do Gonçalves cave entrance **B** cave floor in the inner portion of the cave, where specimens were found **C** water table at the cave lower level **D** adult specimen inside the cave, frontal view **E** adult specimen inside the cave, lateral view **F** diesel pump installed inside the cave in the 70's **G** electric pump installed inside the cave in 2010.

used to remove water from the cave once a week to fill up an external reservoir, hence the water table was persistent. However, in 2010, an electric pump employed by a local farmer for irrigation was installed in the cave (Fig. 4G), leading to a pronounced reduction of at least two meters in the water table inside the cave. Even after the removal of this electric pump (in 2012) the water table reduction continued, since artesian wells were built up outside the cave, so the water demand persisted. During a visit to the cave in August 2013, a major reduction in the water level was observed (approximately 3 m compared to the “pristine” level – observed in the 1990s). In 2013, specimens of *I. leilae* sp. nov. were no longer found in the upper level of the cave (that was extremely dry), being only observed on the moist substrates of the lower

level, even though, in lower densities when compared to the first observations. In December 2018, in a visit to the cave, the whole water table was no longer observed, so previously inaccessible areas were explored, revealing the cave to be much longer than previously thought, and only a few cave-restricted species were observed. From the 22 previously observed troglobitic species, only two (one specimen of *I. leilae* sp. nov., and one springtail) were observed in some moist areas located deep inside the cave in previously flooded areas. The single individual of *I. leilae* sp. nov. was found in a small piece of wood, apparently attracted by the organic compounds. It is important to mention that this species may be seriously threatened, as other cave-restricted species from the Toca do Gonçalves cave. Urgent intervention by the competent environmental agency is needed to ensure the protection of this hotspot of subterranean biodiversity in South America, especially considering that even the most relevant caves can now be destroyed in Brazil (Brasil 2022).

***Iansaoniscus paulae* sp. nov.**

<http://zoobank.org/4B0755B7-18B1-404D-B6F0-A6F7A86A4EF4>

Figs 5–8

Diagnosis. Cephalon with well-developed lateral lobes with round distal margin; frontal shield rounded, weakly developed; pereonite 1 epimeron without dorsolateral furrow; pereonite 1 and 2 without ventral lobes; antennula distal article with two lateral and one apical aesthetascs; third article longer than second; uropod exopod longer than endopod, longer than distal margin of telson.

Material examined. Holotype: BRAZIL • 1 male (mounted in slide); Bahia state, municipality of Itaeté, Lapa do Bode cave (WGS84 -12.938889, -41.062222); 26th July 2007; RL Ferreira leg.; ISLA 95915. **Paratypes:** • 5 females; same data as holotype; ISLA 95916.

Etymology. The new species is named after Dr. Paula Beatriz Araujo, UFRGS, for her contribution to the knowledge of Brazilian terrestrial isopods.

Description. Maximum length: male, 3 mm; female 5 mm. Body outline as in Fig. 5A, colorless, vestigial eyes (Fig. 5A, B). Cephalon with well-developed lateral lobes, round distal margin; frontal shield rounded, weakly developed (Fig. 5B). Pereonite 1 epimeron without dorsolateral furrow; pereonites 1 and 2 without ventral lobes (Fig. 5C, D); pereonite 3–7 with subquadrangular epimera. Pleonites 3–5 with epimera subquadrangular and directed backwards (Fig. 5A). Telson (Fig. 6A) slightly wider than long with concave sides, acute apex. Antennula (Fig. 5E) with three articles, distal article longer than second article, with two lateral and one apical aesthetascs. Antenna (Fig. 5F, 6B) surpassing pereonite 3 when extended backwards, fifth article of peduncle as long as flagellum, flagellum with three articles; faint suture between second and third, third article longest, aesthetascs on second and third articles. Mandibles (Fig. 6C, D) with molar penicil of 4 branches, left mandible with 2+1 penicils, right with 1+1 penicils. Maxillula (Fig. 6E) outer branch with 4 + 5 teeth (two apically

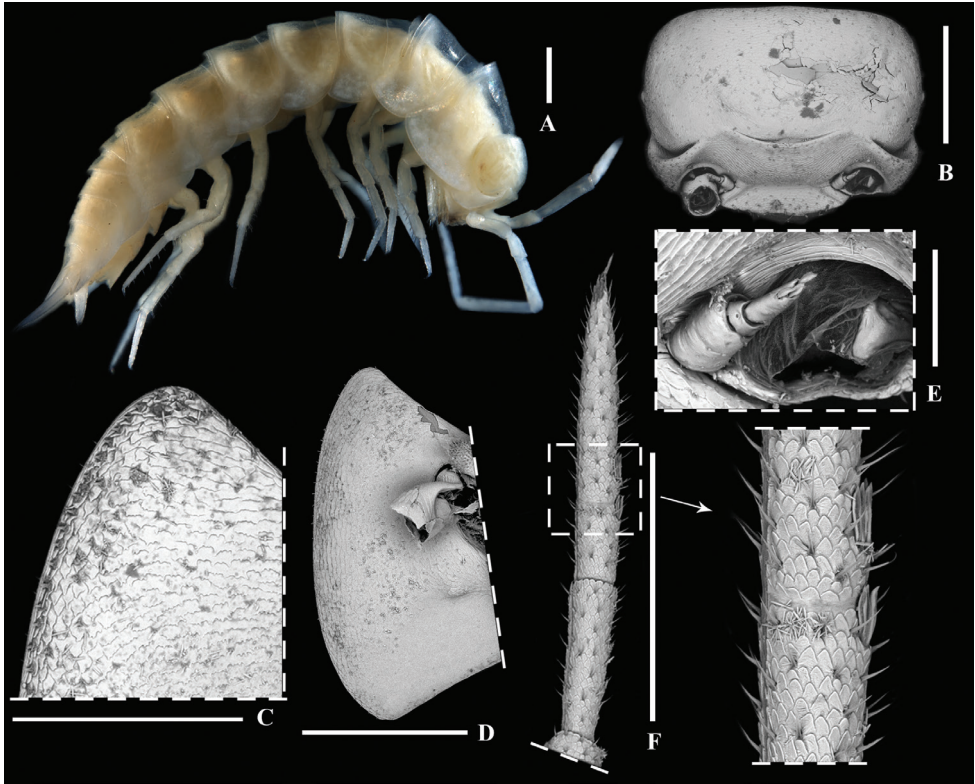


Figure 5. *Iansaoniscus paulae* sp. nov. Female paratype **A** lateral view **B** cephalon, dorsal view **C** pereonite 1, dorsal view **D** pereonite 1, ventral view **E** antennula **F** antennal flagellum. Scale bars: 0.5 mm (**A, B, D, E**); 0.3 mm (**C**); 0.1 mm (**E**).

cleft); inner branch with two penicils. Maxilla (Fig. 6F) with bilobate apex, outer lobe wider than inner lobe, rounded and covered with thin setae; inner lobe bearing thick setae. Maxilliped (Fig. 6G) basis rectangular; palp basal article with two setae distinct in length; endite rectangular, long medial seta, distal margin with two teeth. Pereopods 1–7 (Fig. 7A, B) merus and carpus with sparse setae on sternal margin; carpus 1 with longitudinal antennal grooming brush, distal setae cleft; dactylar organ and ungual seta simple not surpassing outer claw. Uropod (Fig. 7C) protopod distal margin and exopod proximal margin with lateral groove; endopod inserted proximally, exopod 1.4× longer than endopod and distinctly surpassing distal margin of telson.

Male: Pleopod 1 (Fig. 7D) exopod ovoid, twice wider than long; endopod almost four times longer than exopod, distal portion slightly bent outwards, apex with small setae. Pleopod 2 exopod (Fig. 7E) triangular bearing one seta on outer margin, endopod flagelliform, almost three times longer than exopod. Pleopod 3–5 exopods as in Fig. 7F–H.

Habitat and conservation issues. The Lapa do Bode cave consists of a dolomite cave with 1,430 meters of horizontal projection, with conduits of labyrinthine pattern. It is inserted in an upper unit of the Una geological group of carbonate rocks from the

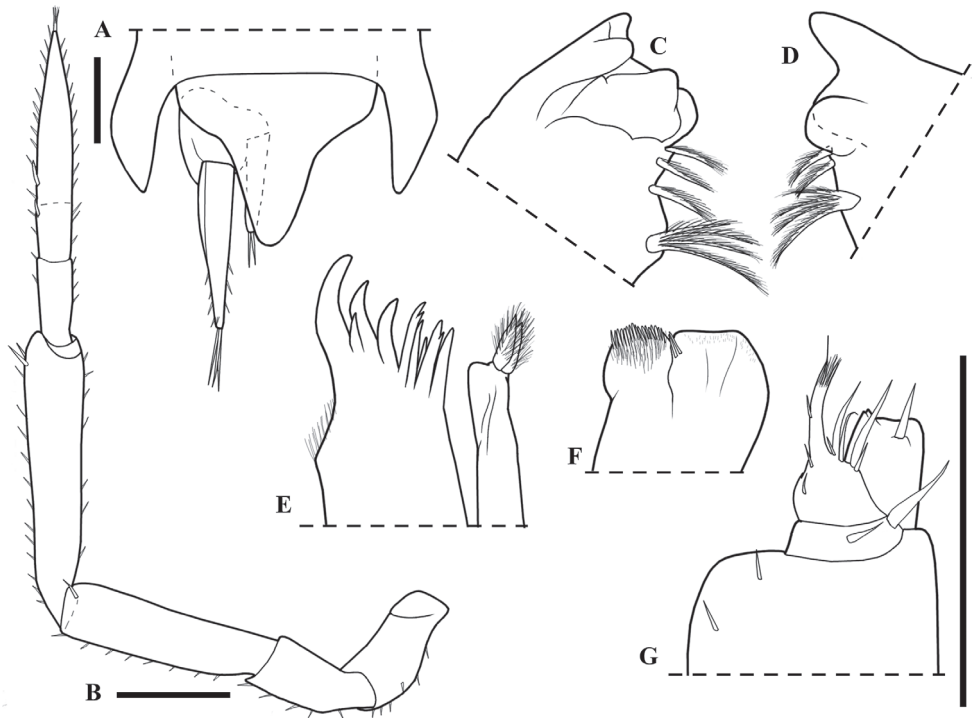


Figure 6. *Iansaoniscus paulae* sp. nov. Male holotype **A** telson and uropod, dorsal view **B** antenna **C** left mandible **D** right mandible **E** maxillula **F** maxilla **G** maxilliped. Scale bars: 0.2 mm.

Salitre Formation (Fig. 9) (Auler 2019). This cave presents five entrances (Fig. 8B, C) inserted in a rock wall parallel to the Una River, which flows close to the entrances (Fig. 8A). Although most of the cave is dry, the cave atmosphere is considerably moist due to water ponds in some inner areas. The temperature inside the cave is around 24.5 °C. The cave has been used for touristic purposes for the last decades, and the areas where individuals of *I. paulae* sp. nov. were found (inner portions of the cave, quite distant from the entrances) are also visited by tourists. They were observed walking on moist soil (Fig. 8D) and in some bat guano piles. Although the touristic activities apparently have not been altering intensively the cave features, there is concern that it could impact the species, especially by trampling (by visitors) due to the reduced size of the specimens and absence of structures guiding visitors to walk in specific pathways. The main available food resource is bat guano, that are concentrated in the inner chambers of the cave. It is worth mentioning that the Lapa do Bode cave presents ten cave-restricted species, among springtails, harvestmen, spiders, a beetle, a millipede, a silverfish, a paligrade and a fish. However, only three of those species are currently described: the carabid beetle *Coarazuphium cessaïma* (Gnaspini, Vanin & Godoy, 1998); the pholcid spider *Metagonia diamantina* (Machado, Ferreira & Brescovit, 2011); and the catfish *Rhamdiopsis krugi* (Bockmann & Castro, 2010).

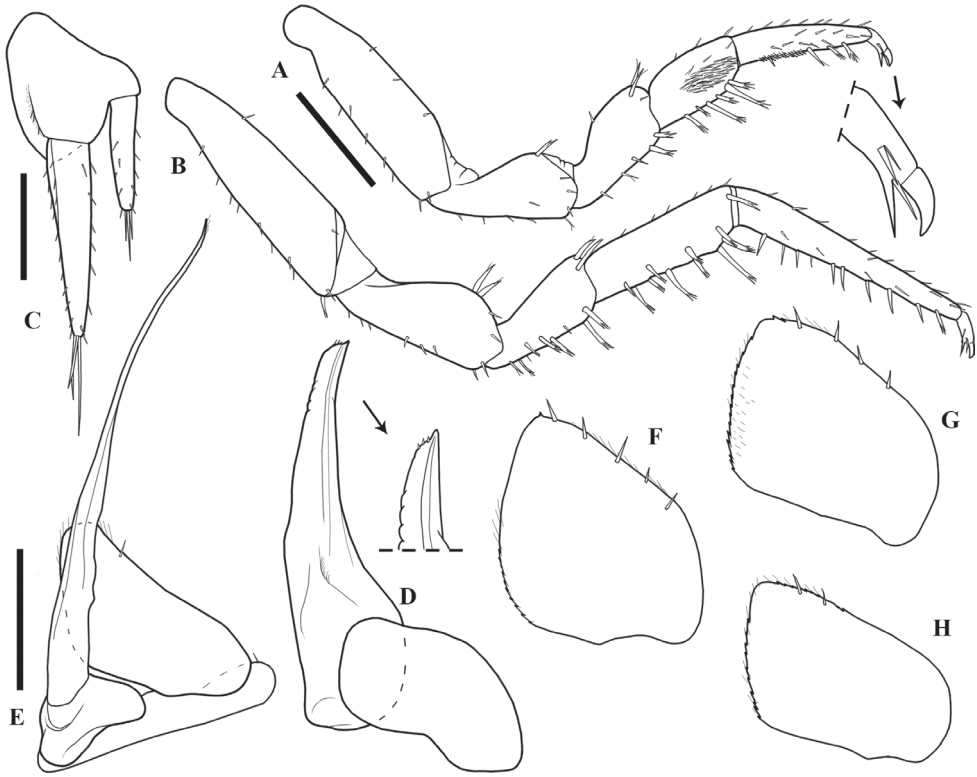


Figure 7. *Iansaoniscus paulae* sp. nov. Male holotype **A** pereopod 1 **B** pereopod 7 **C** uropod **D** pleopod 1 **E** pleopod 2 **F** pleopod 3 exopod **G** pleopod 4 exopod **H** pleopod 5 exopod. Scale bars: 0.2 mm.

Discussion

Taxonomy

The shape of cephalon is a unique feature in *Iansaoniscus* species, however it presents great phenotypic plasticity. *Iansaoniscus leilae* sp. nov. and *I. georginae* show similar patterns such as the frontal shield triangular with frontal margin directed upwards, lateral lobes weakly developed and antennal sockets depressed; while *I. paulae* sp. nov. and *I. iraquara* show well-developed lateral lobes with round margins and frontal shield rounded, weakly developed. The two new species are similar to *I. iraquara* due to pereonites 3 to 7 with subquadrangular epimera, maxillulae outer endite with 4+5 teeth (2 of them apically cleft) and absence of dorsolateral furrow on pereonite 1 epimera. However, while *I. iraquara* and *I. leilae* sp. nov. present ventral lobes on pereonites 1 and 2, such structures are absent in *I. paulae* sp. nov. In *I. georginae* the dorsolateral furrow and lobes are present, but weakly developed, while the maxillulae outer endite presents 4+6 teeth (2 apically cleft). Differently from the other three species, *I. georginae* presents subquadrangular epimera from pereonite 2. Regarding the telson shape, *I. iraquara*, *I. georginae* and *I. paulae* sp. nov. share the condition of a narrowly

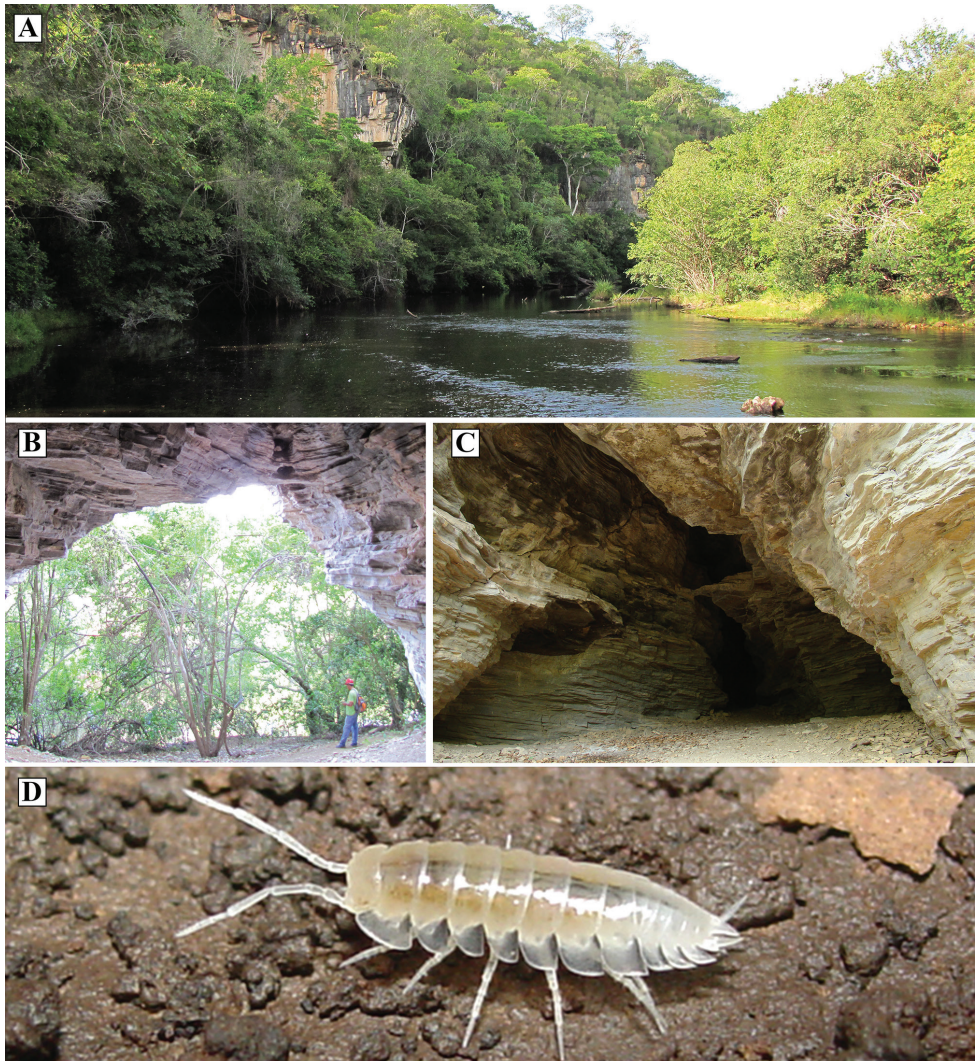


Figure 8. *Iansaoniscus paulae* sp. nov. habitat **A** limestone bordering the Paraguaçu river, where the Lapa do Bode cave's entrance is located **B** one of the entrances of Lapa do Bode cave **C** a secondary entrance of Lapa do Bode cave **D** adult specimen inside the cave.

rounded apex, which is acute in *I. leilae* sp. nov. Another difference among the species is that pleopod 1 exopod may present two conditions: ovoid (*I. iraquara*, *I. leilae* sp. nov. and *I. paulae* sp. nov.), and subquadrangular (*I. georginae*). In relation to eyes, such structures are absent in *I. iraquara*, *I. georginae* and *I. leilae* sp. nov., but present and vestigial in *I. paulae* sp. nov. Moreover, the *Iansaoniscus* species present distinct patterns of aesthetascs in the antennula distal article: *I. georginae* (four lateral plus one apical pair), *I. leilae* sp. nov. (two lateral plus two apical), *I. paulae* sp. nov. (two lateral plus one apical) and *I. iraquara* (missing antennule in the description). Additionally,

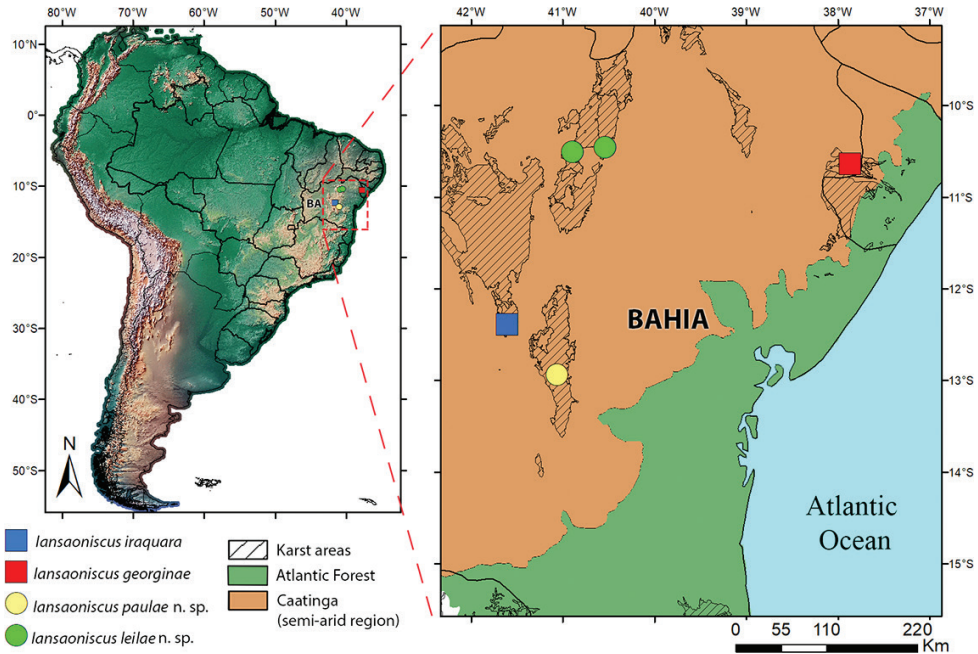


Figure 9. Geographical distribution of *Iansaoniscus* species, with Brazilian biomes and the karst areas delimited in the state of Bahia.

distinct patterns are observed regarding the number of branches in the mandible molar penicil: *I. iraquara* (5 to 7), *I. georginae* (6 to 7), *I. leilae* sp. nov. (7 to 8), *I. paulae* sp. nov. (4). The proportion of uropods exopod and endopod also varies among the species, with exopod as long as endopod in *I. iraquara*; exopod shorter than endopod in *I. leilae* sp. nov.; and exopod longer than endopod in *I. georginae* and *I. paulae* sp. nov. (being longer in *I. paulae* sp. nov. 1.4×).

Ecology and distribution

Until the discovery of *Iansaoniscus* species in the Caatinga biome (semi-arid region) (Fig. 9), the family was believed to be restricted to the Atlantic Forest (Campos-Filho et al. 2018; Cardoso et al. 2018). The other three genera occur in forests along the coast of Brazil, mainly associated with vegetation and/or leaf litter (Vandel 1963; Lemos de Castro 1973; Campos-Filho et al. 2017). Until then, few collection efforts had been made in more arid areas of Brazil, which conditions are not very suitable for the occurrence of isopods in general. In this sense, *Iansaoniscus*, which are restricted to caves in the only Brazilian semi-arid biome (Caatinga), are supposed to represent a geographical relict.

The xeric conditions of Caatinga currently act as a limiting barrier to the dispersion of many hygrophilic components of fauna, such as isopods (Fernandes et al.

2016). However, Wang et al. (2004) dated speleothems and travertine deposits from the Bahia state and estimated 210,000 years of record of wet periods in northeastern Brazil caused by the displacement of the Intertropical Convergence Zone to the south. The rainforest distribution was affected during these wet periods, which formed a corridor between Amazonian and Atlantic Forests (Por 1992; Behling et al. 2000). Posteriorly, Cruz et al. (2009) observed an anti-phased relationship between precipitation in northeastern Brazil and the rest of tropical South America during the Holocene. The authors analyzed oxygen isotopic records from a speleothem in northeastern Brazil from the last 26,000 years and concluded that a predominantly wet climate dominated the currently semi-arid Caatinga. In this sense, the called south-eastern/north-western route (Bigarella et al. 1975), or the route across the Caatinga formation of eastern Brazil (Ledo and Colli 2017), seem to have favored many biotic exchanges in the past. Along this pathway and under climatic changes, some species or groups of species may have been isolated in moister areas, such as the subterranean conditions of many caves in Caatinga (Polhemus and Ferreira 2018), culminating in current geographical relicts, such as *Allokoenenia* (Palpigradi) (Souza & Ferreira, 2022) and *Iansaoniscus* species.

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***Coframalaxius bletteryi* gen. et sp. nov. from subterranean habitat in Southern France (Hemiptera, Fulgoromorpha, Cixiidae, Oecleini)**

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Abstract

A new planthoppers genus and species of Cixiidae Oecleini, *Coframalaxius bletteryi* gen. et sp. nov. newly discovered in a cave near Nice in southern France, is described. Molecular analysis confirms the morphology-based classification of *Coframalaxius* as sister to *Trigonocranus* within the Oecleini. Several morphological characters are further discussed. A double-grasping coxo-femoral and femoro-tibial system is regarded as apomorphic for the oecleini taxa and would allow the nymph to firmly grab the roots and rootlets on which it feeds or use to progress in the soil. Wing vein patterns are discussed in the Cixiidae: 1) for the forewings, Oecleini belong to the trifid type of the anterior MP branch, leading to the reinterpretation of some recently described Neotropical species, 2) for the hindwing, four connection types (U-, V-, Y- and I-types) between MP and CuA are described. Oecleini belongs to I-type with a complete fusion of MP₃₊₄ with CuA₁. Although the area where the cave is located is well-studied with respect to its regularly sampled epigeal fauna for many years, the taxon is new to science, highlighting its probable completely hypogean life cycle and leading to consider *Coframalaxius bletteryi* as an eutroglophile species.

Keywords

Cave, ethology, morphology, planthoppers, wax plates, wing venation

Introduction

As obligate phytophagous insects, planthoppers (Hemiptera, Fulgoromorpha) would not be expected to live in caves. However, the root system of the aerial vegetation offers root-feeding planthoppers the opportunity to eventually adapt and evolve in temporary, cyclical or even permanent hypogean conditions. Since the first report by Racovitza (1907), more than 60 planthopper species in five planthoppers families (Cixiidae Spinola, 1839, Delphacidae Leach, 1815, Meenoplidae Fieber, 1872, Kinnaridae Muir, 1925, and Hypochthonellidae China & Fennah, 1952), have been reported living exclusively in the subterranean ecosystems (Hoch 1994; Hoch 2013, and references therein; FLOW 2022). According to their biology, they have been classified as true cavernicolous species or troglobionts, eutroglophiles (species able to maintain permanent hypogean populations), subtroglophiles (species living temporally or cyclically in hypogean conditions) and troglloxenes (species occurring sporadically in a hypogean habitats, unable to establish subterranean stable populations) (Sket 2008; Howarth and Moldovan 2018).

Although several hypogean species have been reported from the Macaronesian islands (Canary Islands, Azores, Cape Verde) and the Balears (Mallorca: unconfirmed record; Racovitza 1907), in continental Europe, true cavernicolous species remain exceptional. One species of Kinnaridae, *Valenciolenda fadaforesta* Hoch & Sendra, 2021, was recently described from two karstic caves of the Iberian Mountain Range in Spain (Hoch et al. 2021). The other two are Cixiidae Cixiini: *Ibleocixius dunae* D'Urso & Grasso, 2009, described from Sicily from a limestone cave (d'Urso and Grasso 2009) and *Trirhacus helenae* Hoch, 2013, from a dolomite cave in Mljet island, Croatia (Hoch 2013). In the adult stage, they all have more or less strong morphological adaptations to underground life and as troglobionts, they are adapted to the stabilize conditions they experience underground.

Only few planthoppers are considered eutroglophiles and subtroglophiles, which are less strongly linked to hypogenous habitats (FLOW 2022). In Europe, another Oecleini monotypic cixiid genus, *Trigonocranus* Fieber, 1875 with the species *Trigonocranus emmeae* Fieber, 1876, should be regarded as a subtroglophilic species (Hoch et al. 2013). It is a rarely collected species known from few localities in northern parts of western Europe and Russia and seems to inhabit the interstitial ground level. Adults often exhibit varying degrees of certain troglomorphies such as reduction of the compound eyes, wings and weak pigmentation (Hoch et al. 2013).

Following Emeljanov's subdivisions (2002), the classification of the Cixiidae was recently reviewed by Luo et al. (2021) who recognized three main lineages (but without formal rank while awaiting further phylogenetical analysis): the oecleine lineage (including Duiliini Emeljanov, 2002, Cajetini Emeljanov, 2002, Stenophlepsini Metcalf, 1938, Oecleini Muir, 1922, and Bothriocerini Muir, 1923 downgraded from subfamilial rank), sister to a pentastirine (with Pentastirini Emeljanov, 1971 and perhaps Borysthenini Emeljanov, 1989, downgraded from subfamilial rank) and

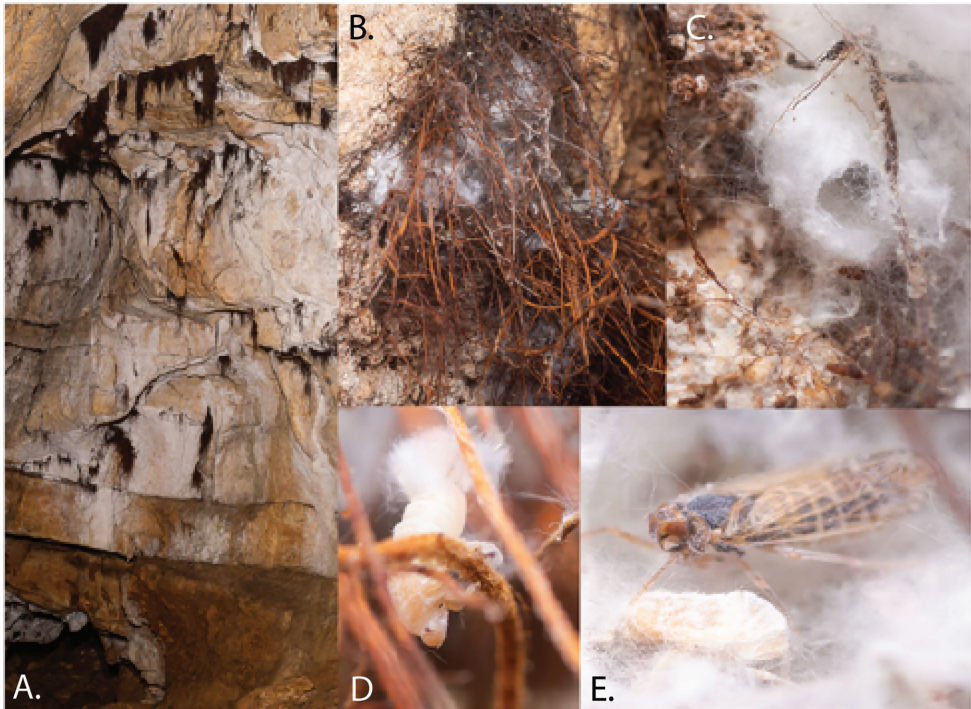


Figure 1. *Coframalaxius bletteryi* in its natural habitat **A** cave with roots **B** roots with cixiid nests characterized by the waxy filaments left by the nymphs **C** waxy galleries where nymphs and adults were observed **D** fifth instar nymph feeding on rootlet **E** adult and nymph inside nest.

a cixiine lineage (including all other cixiid tribes). Three tribes were not addressed by Emeljanov (2002): Gelastocephalini Emeljanov, 2000, Mnemosynini Emeljanov, 1992 and Benarellini Emeljanov, 1989). In this paper, we describe a new genus of Cixiidae in the tribe Oecleini, for a new species recently discovered in a cave in southern France. A molecular analysis confirms its placement in the Oecleini. This species has the classic nymphal troglomorphies present in most species of the family and adults look like typical epigeal species. However this planthopper has never been observed in any habitat above ground, even though the area where the cave is located has been well investigated with respect to its epigeal fauna over many years. Accordingly, the ecological classification of the new species is discussed.

Materials and methods

Specimens were collected by hand with an aspirator and killed in cyanide jars. All specimens are stored dried on cardboard in the Muséum national d'Histoire naturelle, Paris, France (MNHN). Each label under a specimen is reported between brackets:

[label1][label2]... For descriptive purposes, entire nymphs or adult abdomens were boiled in 10% NaOH solution for a few minutes. Residual endodermic soft tissues were removed in distilled water before transferring the whole abdomen into glycerin for dissection and observation. Dissected nymphs, abdomen parts and genitalia were stored under their related specimens in genitalia vials in a drop of glycerin for final conservation. Preparation and observation of specimens were done under a Leica MZ12.5 stereo microscope. Photos were taken either with the adapted module Leica IC90E and Leica Acquire software (version 2.4.6 Build 9112), either with a Canon EOS 6D with a Macrolens Canon EF 100 mm f/2.8, and then assembled with the software Helicon focus 6.

Morphological terminology for male genitalia follows Bourgoïn (1988), for female genitalia Bourgoïn (1993), and forewing venation Bourgoïn et al. (2015). Terminology of vertex is adapted from Loecker (2014: Fig.1) and follows Fig. 2. The metatibiotarsal formula $IT-(aTd)/aI/aII$ corresponds to the number of lateral (IT) and apical teeth (aT) on the metatibia, eventually with a diastema (d), the number

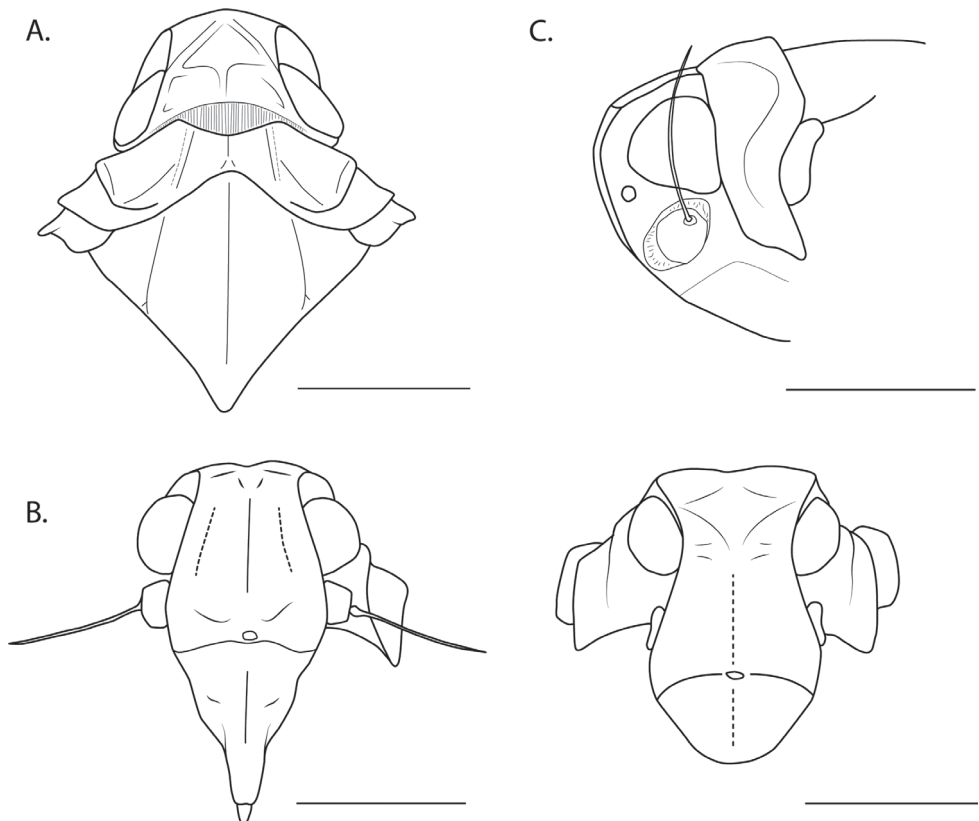


Figure 2. *Coframalaxius bletteryi* gen. et sp. nov. head capsule **A** dorsal **B** frontal **C** dorsofrontal and lateral view. Scale bar: 0.5 mm.

of apical teeth (aI) on metatarsomere I, and the number of apical teeth (aII) on metatarsomere II.

Total genomic DNA was extracted from legs muscle tissue using the Qiagen DNEasy kit (Qiagen, Inc., Valencia, CA, USA). Analyses were conducted on partial sequences of *18S rDNA* (1939 bp; first third of the gene not sequenced), *28S rDNA* (D3-D10; 3057 bp), cytochrome oxydase I (*COI*; 1239 bp), cytochrome b (*Cytb*; 426 bp), and histone 2A (*H2A*; 306 bp). Oligonucleotide primers used for polymerase chain reaction (PCR) amplification are listed in Table 1. The full dataset includes 37 taxa: 3 delphacids as out-group taxa and 34 ingroup taxa representing all three subfamilies of the Cixiidae (Borysteninae, Bothriocerinae, Cixiinae). All species available to us from the oeclinian lineage *sec.* Luo et al. 2021, were included; 29 species were directly available from GenBank of which we added 8 new samples. All related registration numbers in GenBank are provided in Table 2.

Phylogenetic reconstruction using maximum likelihood (ML) was generated in Phylip formats using PhyloSuite V1.2.1 (Zhang et al. 2020). The best partitioning scheme and replacement model were selected by PartitionFinder V2.0.0 (Lanfear et al. 2016), resulting in 9 partitions fitting 6 best models (Table 3). Maximum likelihood (ML) analysis were carried out with IQTREE V1.6.8 (Guindon et al. 2010; Minh et al. 2013; Nguyen et al. 2015). The resulting topology was prepared with iTOL v5 (Letunic and Bork 2021).

Table 1. Primer sequences used for the molecular analysis.

Primer	Sequence (5' – 3')	Primer Source
<i>COI</i>		
2183 (F)	CAACATTATTTTGATTTTGTGG	Simon et al. (1994)
UEA8 (R)	AAAAATGTTGAGGGAAAAATGTTA	Lunt et al. (1996)
<i>Cytb</i>		
CB1 (F)	TATGTACTACCATGAGGACAAATATC	Jermiin and Crozier (1994)
CP2 (R)	CTAATGCAATAACTCCTCC	Harry et al. (1998)
<i>18S rDNA</i>		
574 (F)	GCCGCGGTAATTCCAGCT	Bourgoin et al. (1997)
E21 (R)	CTCCACCAACTAAGAACGG	
18S–mid (F)	GATACCGCCCTAGTTCTAACC	
2200 (R)	CGGCAGGTTACCTACGG	
<i>28S rDNA</i>		
Ai (F)	GACCCGTCTTGAAACACG	Litvaitis et al. (1994)
D4D5r (R)	GTTACACACTCCTTAGCGGA	Belshaw and Quicke (2002)
EE (F)	CCGCTAAGGAGTGTGTAA	Cryan et al. 2000
MM (R)	GAAGTTACGGATCTARITTG	Dietrich et al. 2001, as 'fragment IV
Lalt (F)	CCTCGGACCTTGAAAATCC	
Galt (R)	TGTCTCCTTACAGTGCCAGA	
V (F)	GTAGCCAAATGCCTCGTCA	Cryan et al. 2000
X (R)	CACAATGATAGGAAGAGCC	
<i>H2A</i>		
F1 (F)	TGTCTGGYCGCGGCAARGG	Cryan & Urban, 2011
R1 (R)	ACGGCCTGGATGTTGGGCA	

Table 2. Investigated species, with their main distribution and registration numbers of sequenced genes. New sequences are marked with (*).

Species	Distribution	COI	Cytb	18S	28S	H2A
Cixiidae						
<i>Achaemenes interspersus</i> Jacobi, 1907	Madagascar	EU183598	–	EU183575	EU183704	–
<i>Borbonomyndus pandanicola</i> Attié, Bourgoïn & Bonfils, 2002	Reunion Island	EU183593	–	EU183571	EU183735	–
<i>Borysthene</i> sp. *	China: Hunan	ON079066	ON113340	ON087638	ON130260	–
<i>Bothriocera eborea</i> Fennah, 1943	US Virgin Island	EU645971	–	DQ532511	DQ532591	JN797395
<i>Bothriocera</i> sp.1	Martinique	EU183603	EU183642	EU183577	EU183670	–
<i>Bothriocera</i> sp.2	Belize	EU183604	–	EU183581	EU183675	–
<i>Coframalaxius bletteryi</i> gen. et sp. nov. *	France	ON087562	ON113342	ON087640	ON231299	ON101633
<i>Colvanalia</i> sp. *	China: Zhejiang	–	OK169309	MW306541	–	–
<i>Cixius bicolor</i> Matsumura, 1914 *	China: Taiwan	–	OK169315	MW306536	MW306556	ON101626
<i>Cixius</i> sp. *	China: Shaanxi	MW291679	ON152767	MW306516	MW306544	OK169342
<i>Colvanalia taffini</i> Bonfils, 1983	Vanuatu	EU183613	–	EU183560	EU183690	–
<i>Haplaxius crudus</i> (Van Duzee, 1907)	USA (FL)	EU183606	–	EU183553	EU183680	–
<i>Haplaxius deleter</i> (Kramer, 1979)	Costa Rica	EU183605	EU183631	EU183552	EU183679	–
<i>Haplaxius delta</i> (Kramer, 1979)	Costa Rica	MT900602	–	MT892908	–	–
<i>Haplaxius douguabshi</i> Bahder et al., 2020	Costa Rica	MT080284	–	MT002395	–	–
<i>Haplaxius lunatus</i> (Van Duzee, 1909)	USA (FL)	OM264285	–	OM258692	–	–
<i>Haplaxius pictifrons</i> (Stål, 1802)	Costa Rica	MT946292	–	MN200098	–	–
<i>Haplaxius</i> sp.	Costa Rica	MW086873	–	MW086509	–	–
<i>Haplaxius skarphion</i> (Kramer, 1979)	Mexico	–	–	EU183570	EU183682	–
<i>Meenocixius virescens</i> Attié, Bourgoïn & Bonfils, 2002	Reunion Island	EU183614	EU183639	EU183572	EU183736	–
<i>Myxia baynardi</i> Bahder & Bartlett, 2021	Costa Rica	MT900604	–	MT892909	–	–
<i>Myxia belinda</i> Bahder & Bartlett, 2020	Costa Rica	MT900605	–	MN200096	–	–
<i>Myxia hernandezi</i> Bahder & Bartlett, 2021	Costa Rica	MZ234085	–	MZ262449	–	–
<i>Nymphocixia caribbea</i> Fennah, 1971	Cuba	EU183615	–	EU183561	–	–
<i>Nymphocixia unipunctata</i> Van Duzee, 1923	USA (FL)	OM264284	–	OM258690	–	–
<i>Oecleopsis tiantaiensis</i> Guo, Wang & feng, 2009 *	China: Hunan	MW291684	–	MW306535	MW306590	–
<i>Oecleus mackaspringi</i> Myrie et al., 2019	Jamaica	MN488999	–	MN422261	–	–
<i>Oecleus peripictus</i> Van Duzee, 1929	USA(AZ)	–	–	JQ982515	JQ982532	–
<i>Oecleus productus</i> Metcalf, 1923	USA	–	EU183647	–	EU183719	–
<i>Oecleus</i> sp.2	USA (UT)	EU645972	–	DQ532512	DQ532592	–
<i>Oecleus</i> sp.1	Belize	–	EU183649	–	EU183662	–
<i>Oliarus</i> sp. *	China: Guizhou	MW291688	–	MW306513	MW306564	–
<i>Pintalia alta</i> Osborn, 1935	US Virgin Island	–	–	AY744804	AY744838	–
<i>Trigonocranus emmae</i> Fieber, 1876 *	Belgium	–	–	ON260952	–	–
Delphacidae						
<i>Asinaca clavicornis</i> (Fabricius, 1794)	Kyrgyzstan	AF304409	–	HM017281	HM017389	–
<i>Kelisia curvata</i> Beamer, 1954	USA(PA)	–	–	HM017235	HM017343	–
<i>Ugyops stigmata</i> (Crawford, 1914)	Belize	HM017501	–	HM017301	HM017409	–

Table 3. Best partitioning schemes and models for maximum likelihood (ML) analysis.

Genes/codons in partition	Model in IQtree
COI, 18S and 28S	GTR+I+G
nt1 of H2A	GTR+I
nt1 of Cytb	GTR+G
nt2 and nt3 of Cytb	K81UF+I+G
nt3 of H2A	K81UF+G
nt2 of H2A	K81

Taxonomy

Hemiptera Linnaeus, 1758

Cixiidae Spinola, 1839

Cixiinae Spinola, 1839

Oecleini Muir, 1922

***Coframalaxius* Bourgoïn & Le Cesne, gen. nov.**

<http://zoobank.org/978AA57A-FCE2-45DC-8512-E555F327B1B>

Type species. *Coframalaxius bletteryi* Le Cesne & Bourgoïn (by present designation and monotypy).

Etymology. Arbitrary combination of the first syllabus of author (T. Bourgoïn) four grandsons with suffix -xius from *Cixius*, type genus of the family Cixiidae.

Diagnosis. Small cixiids, in habitus resembling *Trigonocranus* Fieber, 1875, but can be distinguished from the latter by the combination of the following characters: 1) pygofer longer in lateral view, expanded in a triangular lobe on its latero-posterior margin, 2) anal tube with proximal pair of lateroventral teeth, 3) posterior part of gonostyli wider and 4) aedeagus with one internal spine-like process. Female pygofer elongated, without wax plates.

Description. Head capsule. Vertex with posterior compartment sub-rectangular, anterior compartment triangular; subapical carina straight weakly marked, apical carina well distinct, median carina weak vanishing at subapical carina level; in lateral view, slightly surpassing lateral carina. Frons wider at ventral level of antennae in frontal view; frontoclypeal suture slightly arched dorsally, median carina weak, distinct only in dorsal part and not reaching median ocellus; in lateral view, regularly convex, slightly surpassing laterofrontal carina. Postclypeus with lateral margins slightly concave in basal 1/3; in frontal view, median carina weak in ventral 2/3. Anteclypeus lacking median carina. Compound eye thinly elongated in dorsal view. Antennal socket wide, emarginated, almost touching ventral margin of compound eye; scape short, pedicel globular with distinct transversal margin in frontal view, flagellum with basal swelling well developed, almost five times as long as pedicel, surpassing in length the level of lateral side of abdomen (Fig. 2). Lateral ocelli present, separated from compound eye by

1X their length. Labium almost reaching metacoxae; apical segment $1/4$ length of subapical one, slightly thinner medially, proximal segment half as long as subapical one.

Thorax. Prothorax anterior margin widely roundly concave (Fig. 2); posterior margin more sharply roundly concave (Fig. 2); median carina present, not reaching posterior margin; postocular carinae well distinct, not meeting posterior margin and running subapically to its ventral margin in frontal view (Fig. 2B, C). Mesonotum tricarinate with median and lateral carinae. Forewing elongated; stem $ScP+R+MP$ slightly longer than basal cell length, forking at $1/4$ of forewing length, before level of $PCu+A1$ fusion; anterior and posterior margins subparallel. $C1$ cell distinctly curved; anterior branch of MP (MP_{1+2}) forking twice: in MP_1 and MP_2 , then MP_{1a} and MP_{1b} . Posterior branch MP_{3+4} single, unforked. $C5$ cell short, diamond-shaped, ending with CuA_1+CuA_2 fused; icu reaching apex of clavus (Fig. 3A). Hindwing with MP and CuA connecting in I-type (Fig. 3C).

Hindleg with metatibia laterally unarmed, with 6 apical teeth separated in two groups of 3 by a wide diastema, and outermost tooth largest; first metatarsomere elongate, not dilated apically, with 8–9 apical teeth; second metatarsomere with (7–8) apical teeth, without setae under the first one or two teeth on each side, with one long straight setae under the three to five medium teeth; metatibitarsal formula: $0-(3d3)/(8-9)/(7-8)$

Male genitalia. Anal tube symmetrical, with an anterior pair of lateroventral conspicuous hook-like spines. Pygofer symmetrical, dorsocaudally produced into a triangular lobe; suspensorium developed, X-shaped, attached to ventral margin of anal

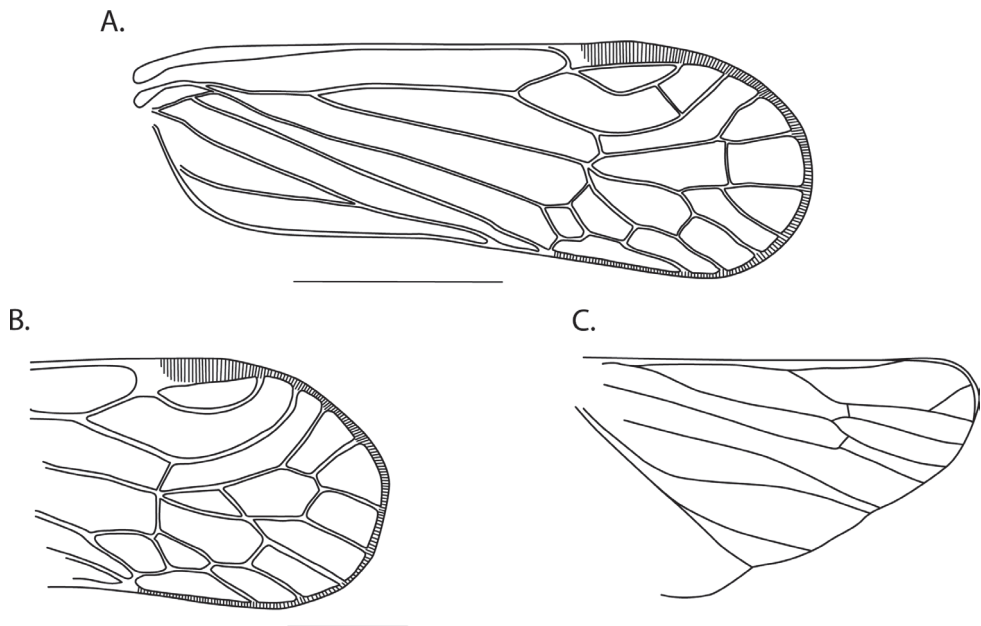


Figure 3. Wings of *Coframalaxius bletteryi* gen. et sp. nov. **A** tegmina venation pattern **B** variation of tegmina venation **C** hind wing venation pattern. Scale bars: 1 mm (**A**, **C**); 0.5 mm (**B**).

tube (Fig. 4). Male copulatory organ with periandrium tube-like, elongated, bearing spines and processes. Aedeagus s.s. very short, endosoma not or very shortly developed. Gonostyli bilaterally symmetrical, with proximal portion slender, apically developed into a spoon-shaped extension with a nearly pentagonal outline, directed dorsally.

Female genitalia of orthopteroid type, sword-shaped ovipositor, following paired hemisternite VII medially divided by a membranous portion (Fig. 5). Pygofer of elongated type without ventral wax plate. Gonoplac fused, apically separated. Ductus receptaculi regular, short, not developed in a helix-twirled structure (Fig. 5C).

Nymphs. Two short laterometatibial spines in 5th instar (Fig. 6). With abdominal paired tergal wax plates on tergites VI to VIII divided into 6 subplates separated by one sensory pits, those ones ranged in row in subanterior position (Fig. 6). In instars 3 to 5: procoxa with one sharp anterior process bearing small (sensorial?) dark triangular microcuticular sculptures; profemur with a conspicuous latero-extern process bearing a row of short sensorial setae-like structures, protibia short distally truncate, bearing two tarsomeres.

***Coframalaxius bletteryi* Le Cesne & Bourgoin, sp. nov.**

<http://zoobank.org/BF08E58D-256F-4286-9CEB-4A87A736D384>

Genbank registration. ON087562/ ON113342/ ON087640/ ON231299/ ON101633.

Etymology. Dedicated to Jonathan Blettery who discovered the first specimen during a fieldtrip with the two first authors exploring caves around Nice in the south of France.

Diagnosis. Small species externally similar to *Trigonocranus emmeae* Fieber, 1876, from which it can easily be separated by the triangular areolet of the vertex (versus pentagonal (Emeljanov 2015: 69 and fig.21.6) in *T. emmeae*), but also by the rounded posterior margin of the pronotum (versus angular), the conformation of the male genitalia with thinner spiniform processes also different in number and conformation and particularly by a unique internal distinct process inside the periandrium (Fig. 4).

Color. Compound eyes, post clypeus, areolet, anterior part of prothorax behind vertex and mesonotum black, frons paler and carinae yellowish-brown. Tegmina translucent without color patches, pterostigma and vein pale brown, with setiferous granules darker; veins after nodal line darker. Legs pale brown. Metatibia and metatarsomere teeth black, median spines of metatarsomere II with setae paler.

Male terminalia with anal tube regularly ovoid, in dorsal view more or less regularly convex lateroventrally in lateral view with a pair of lateroventral teeth directed posteroventral. Male genitalia asymmetrical with suspensorium X-like, connected to the perandrium basally shortly wide then distally tube-like; bearing 5 spiniform processes: a very basal and dorsal short straight spiniform process (1), on left side a long proximal spiniform process directed dorsoposteriorly then bent posteroventrally (2), a short acute ventral process (3), an elongate dorsal spiniform process forked at mid length in a short teeth-like (4) and a short internal hook-like process, located inside the

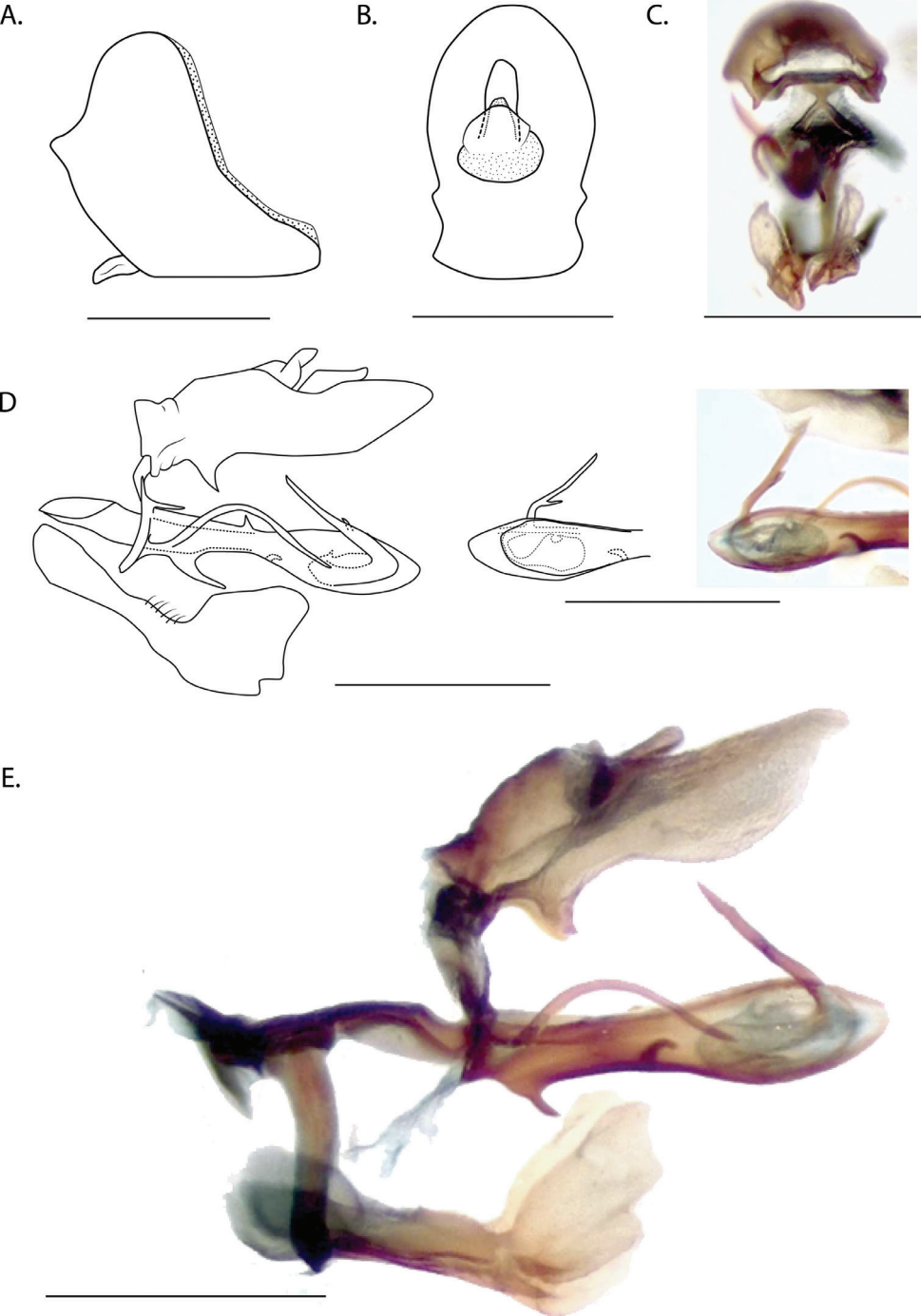


Figure 4. *Coframalaxius bletteryi* gen. et sp. nov. Male genitalia **A** pygofer lateral view **B** anal tube, dorsal view **C** suspensorium **D, E** male genitalia left lateral view **F, G** male genitalia apex, right side. Scale bar: 0.5 mm.

periandrium (5). Gonopore opening large on apical right side, endosoma not visible, probably very weakly developed.

Female terminalia (Fig. 5) with paired sternite VI; each gonocoxa VIII developed in a wide plate slightly bilobed lateroapically; gonapophyses IX fused, slightly larger than gonapophyses VIII; gonapophyses VIII larger proximally in a wide triangular endogonocoxal lobe; gonoplacs longer than gonapophyses and enveloping them.

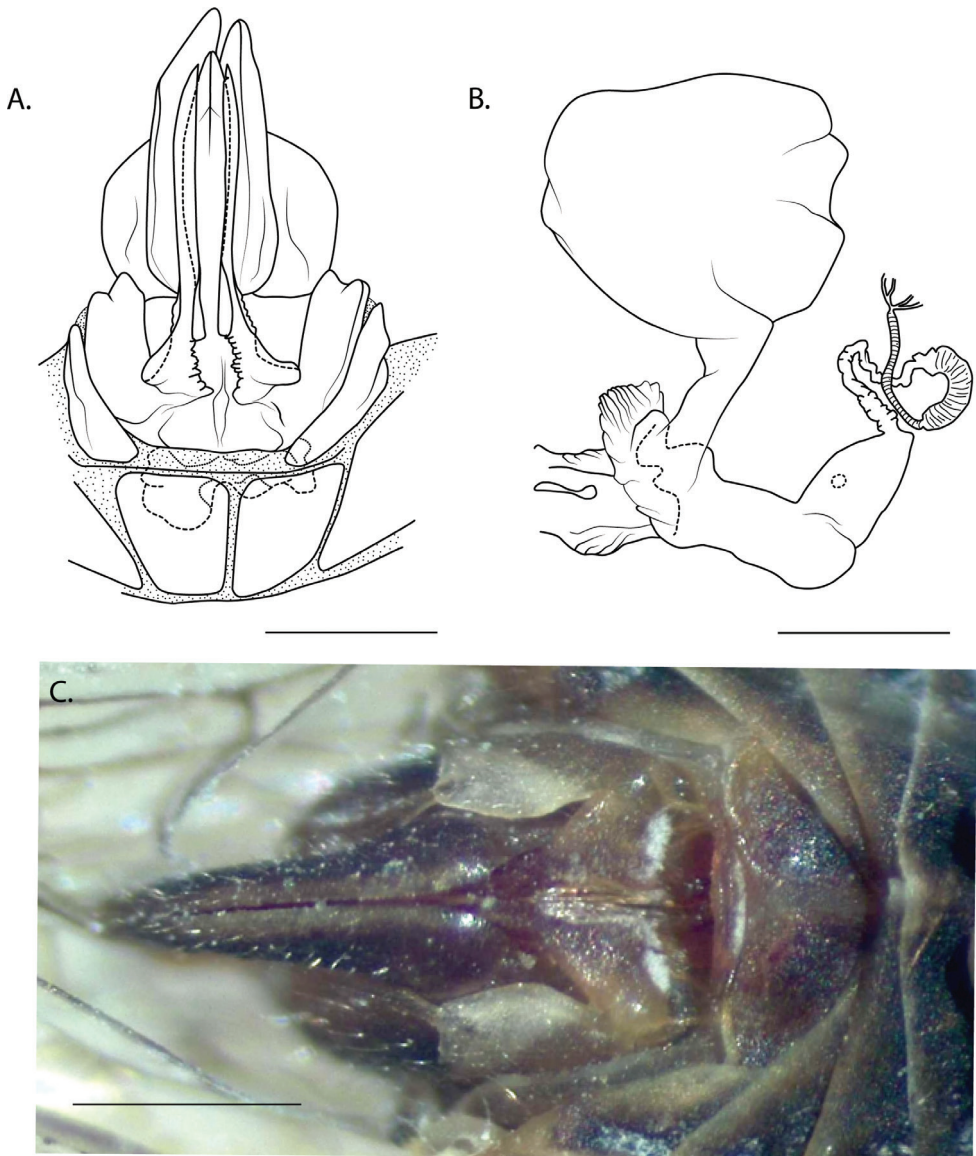


Figure 5. *Coframalaxius bletteryi* gen. et sp. nov. Female genitalia **A, B** ventral view **C** ectodermal internal ducts of female genitalia. Scale bar: 0.5 mm.

Opening of ectodermic internal parts with developed lateral swellings of vestibulum, opening into posterior vagina developed in a strong wrinkled membranous pouch on left side, opening dorsally into the ductus bursae leading to a membranous translucent bursa copulatrix, and laterally into the anterior vagina bent at mid length and ending in the spermatheca; spermatheca with pars intermedialis, half-length of anterior vagina, opening into a diverticulum ductus bent, followed by a long pars intermedialis before vanishing into the the mesodermic glandula apicalis.

Material type. *Holotype* male, pinned, original description: [Grotte de la chèvre d'or; Roquefort-less-Pins; France (06)], [12-VII-2021; J. Blettery rec.], [*Coframalaxius bletteryi* Le Cesne & Bourgoïn sp. nov.; M. Le Cesne det. 2022], [MNHN(EH) 24997].

Paratypes: 1 female, pinned, with genitalia in a separate microvial, original label: [Grotte de la chèvre d'or; Roquefort-les-Pins; France (06)], [*Coframalaxius bletteryi* Le Cesne & Bourgoïn sp. nov.; M. Le Cesne det. 2022], [MUSEUM PARIS; 12-VII-2021; T. Bourgoïn rec.], [Museum Paris; MNHN(EH) 24998] ; 4 females, pinned, original label: [France, 06; Roquefort-les-Pins; grotte de la chèvre d'or],[MUSEUM PARIS; 12-

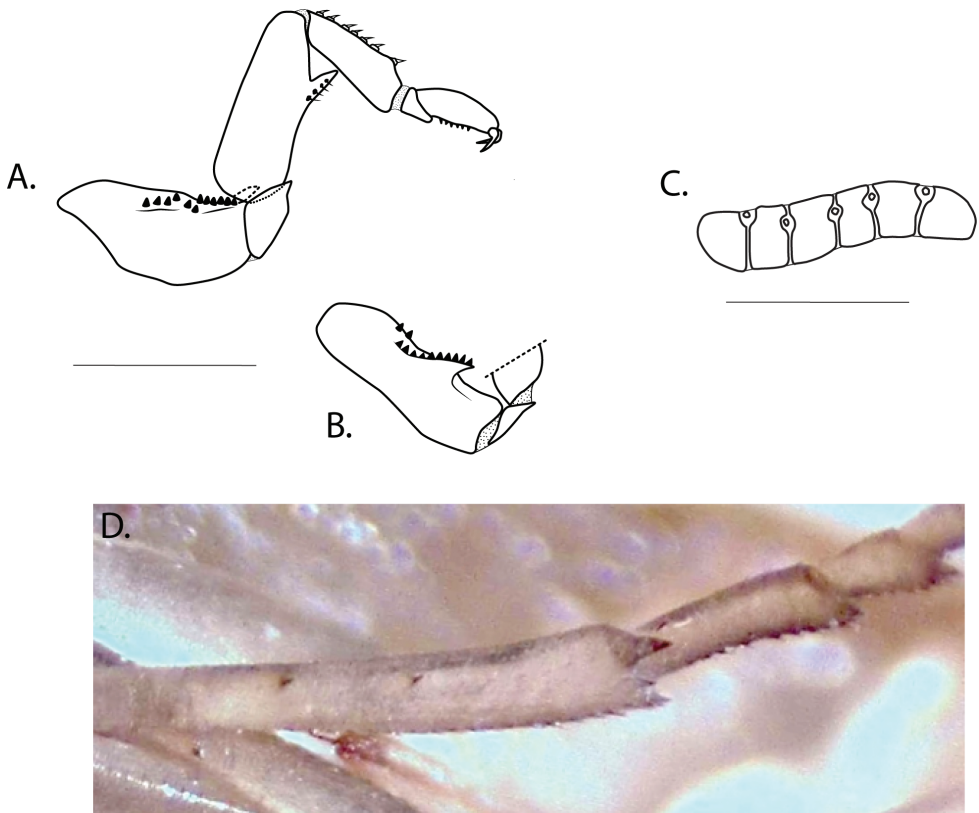


Figure 6. *Coframalaxius bletteryi* gen. et sp. nov. Fifth instar nymph **A, B** proleg, ventral view and anter-oventral view **C** Sternite VI, wax plates. Scale bar: 0.5 mm.

VII-2021; T. Bourgoïn rec.], [*Coframalaxius bletteryi* Le Cesne & Bourgoïn sp. nov.; M. Le Cesne det. 2022], [Museum Paris; MNHN(EH) 25177, 25178, 25179, 25180] ; 2 males, pinned, with genitalia in a separate microvial, original label: [France, 06; Roquefort-les-Pins; grotte de la chèvre d'or],[MUSEUM PARIS; 17-VII-2021; M. Le Cesne rec.], [*Coframalaxius bletteryi* Le Cesne & Bourgoïn sp. nov.; M. Le Cesne det. 2022], [Museum Paris; MNHN(EH) 25181, 25182] ; 3 males, pinned, original label: [France,



Figure 7. *Coframalaxius bletteryi* gen. et sp. nov. Habitus photos.

06; Roquefort-les-Pins; grotte de la chèvre d'or],[MUSEUM PARIS; 12-VII-2021; T. Bourgoïn rec.], [*Coframalaxius bletteryi* Le Cesne & Bourgoïn sp. nov.; M. Le Cesne det. 2022], [Museum Paris; MNHN(EH) 25183, 25184, 25185] ; 3 males, pinned, original label: [France, 06; Roquefort-les-Pins; grotte de la chèvre d'or],[MUSEUM PARIS; 12-VII-2021; J. Blettery rec.], [*Coframalaxius bletteryi* Le Cesne & Bourgoïn sp. nov.; M. Le Cesne det. 2022], [Museum Paris; MNHN(EH) 25186, 25187, 25188].

Other material. Several other nymphs at various instars, pinned.

Type locality. Roquefort-les-Pins, Alpes-Maritimes, France

Phylogeny. *Coframalaxius bletteryi* was sequenced for: COI, Cytb, H2A, 18S and 28S (D3-D5, D6-D7), but only successfully sequenced for 28S (D6-D7) for *Trigonocranus emmae*. Comparison between the two taxa shows significant differences in both total characters and base frequencies, that differs by 37 characters (4.4%) (Table 4). The resulting topology of the molecular analysis (Fig. 8) confirms the placement of *Coframalaxius* into Oecleini as sister to *Trigonocranus*.

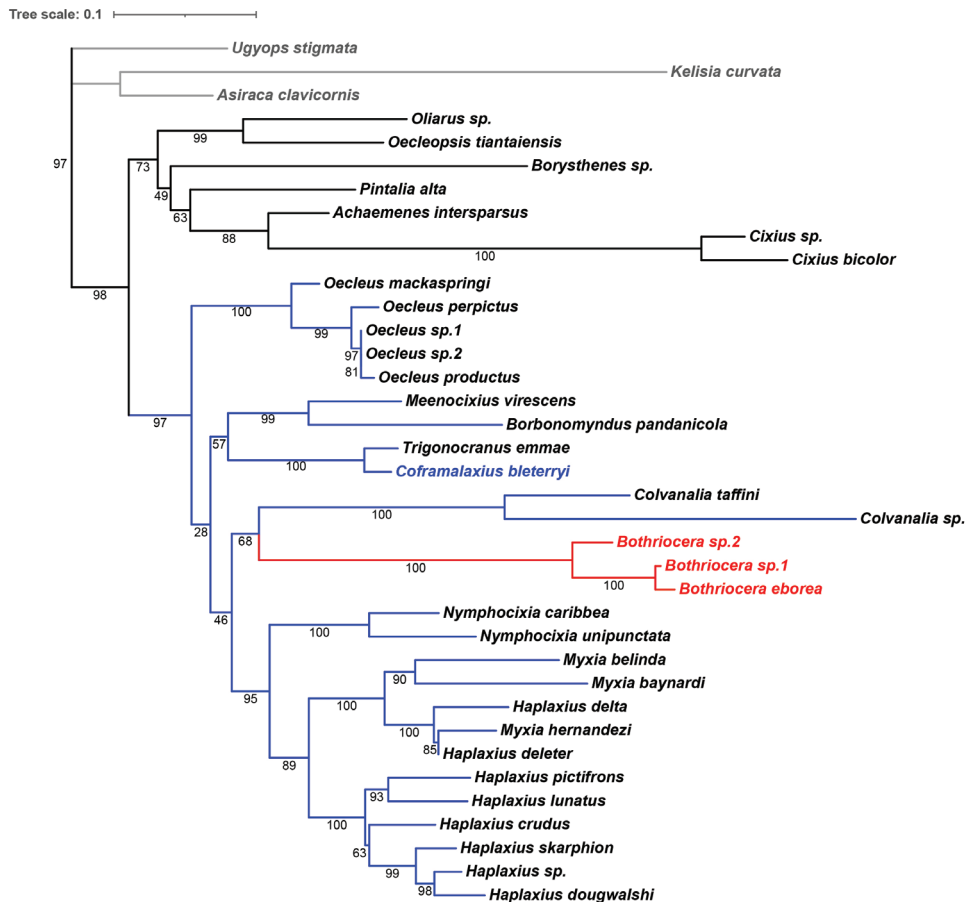


Figure 8. *Coframalaxius* placement within Cixiidae by molecular phylogeny analysis. Out-groups in grey, Oecleini in blue, Bothriocerini in red. Node labels provide UFBoot support values.

Table 4. Summary of the sequence information of 28S (D6-D7) of *Trigonocranus* and *Coframalaxius*.

28S (D6-D7)	Total characters	Base frequencies (%)			
		A	C	G	T
<i>Trigonocranus emmeae</i>	837	21.4	25.9	32.9	19.8
<i>Coframalaxius bletteryi</i>	830	21.6	25.1	33.1	19.4

Discussion

1. *Coframalaxius* classification in the Cixiidae Oecleini

According to Emeljanov's 2002 classification, *Coframalaxius* can be excluded from the pentastirine and cixiine lineage (Luo et al. 2021) based on the presence of paired sternite VIII in males (*versus* unpaired), icu joining apex of clavus (*versus* displaced distal to apex of clavus), the laterally unarmed metatibia with the presence of a metatibial diastema (*versus* a more or less regular line of teeth), and an elongated female genitalia (*versus* reduced or short). All these characters, together with the head capsule conformation distinctly different from the Bothriocerini type, places the new genus within the Oecleini.

The placement of *Coframalaxius* in Oecleini is also confirmed by the molecular phylogeny analysis (Fig. 8) which puts it relatively close to its basal node in a strongly supported Palaearctic clade with *Trigonocranus*, both being sister to a La Reunion clade grouping *Borbonomyndus* Attié, Bourgoin & Bonfils, 2002, and *Meenocixius* Attié, Bourgoin & Bonfils, 2002. If the oecleine lineage appears well supported, the basal branching of the different groups of taxa (including Bothriocerini) remain weak and need further analysis, which is beyond the scope of this paper. Interestingly, the separation between the two genera *Haplaxius* Fowler, 1904 and the recently described *Myxia* Bahder & Bartlett, 2019, also needs more investigation. However, only 9 out of 25 currently recognized oecleine genera are present in the phylogenetic analysis. In particular, the genus *Myndus* Stål, 1862 is not represented, whereas, with its worldwide distributed 81 species (Bourgoin, 2022), it very probably represents a paraphyletic unit.

Our phylogenetic analysis posits a paraphyletic Oecleini, including Bothriocerini, rather than a sister relationship between the two tribes as suggested by Emeljanov (2002). However, the phylogenetic relationships of the oecleine lineage and more specifically within the Oecleini (*s. l.*) will be addressed in another paper (Luo et al. in prep).

2. Morphological characters in support of placement of *Coframalaxius* into Oecleini

Nymphal prolegs

Myers (1929) first described the fossorial prolegs of the fifth instar of *Bothriocera signoretti* Stål, 1864 as did Wilson and Tsai (1982) and Wilson et al. (1983) of the fifth instars of the oecleines *Haplaxius crudus* (Van Duzee, 1907) and *Oecleus borealis* Van Duzee, 1912. Emeljanov (2002) mentioned the "thick and dentate fossorial forelegs" in nymphs as a possible characteristic of Bothriocerini, Oecleini, and probably Cajetini (because of their

unique thick adult forelegs). Subsequently with his figure (Emeljanov 2002: fig. 12), he implied inclusion of *Stenophepsiini* in this group of tribes, but not of *Duiliini*.

Coframalaxius confirms this very special character as a probable morphological synapomorphy of the tribes mentioned by Emeljanov (2002). The structure of the prolegs was compared to the fossorial prolegs of cicada nymphs. We think the term “fossorial” is not appropriate, but the term raptor which Myers (1929) also mentioned is even less so. Based on the morphology of the proleg we suggest that it is more likely a double-grasping system (1) between the coxal apophysis and the femur, and (2) between the femoral apophysis and the tibia. This double-grasping mode would allow the nymph to firmly grab the roots and rootlets on which it feeds or use it to progress in the soil.

Forewing venation

In the Fulgoromorpha ground pattern (Shcherbakov, 1996; Bourgoïn et al. 2015) the media vein of the tegmina forks once at the nodal line into an anterior branch and a posterior branch, both respectively forking again into MP_1 , MP_2 , and MP_3 , MP_4 (Fig. 9A). As mentioned by Emeljanov (2002), a five MP branches probably belongs to the ground plan of the Cixiidae, but occurring in two different patterns. In most cixiids, the trifid anterior branch of the media (M_{1+2}) is realized by the first forking in MP_1 and MP_2 generally occurring between the nodal line and the submarginal line and the second forking of MP_1 into MP_{1a} and MP_{1b} at or after the submarginal line (Fig. 9B). The fork of the posterior branch of the media (MP_{3+4}) into MP_3 and MP_4 is generally part of the submarginal line. From this basic cixiid schema, a clear distinctive one with still five MP terminals but with a bifid anterior media branch (MP_1 , MP_2) and a trifid posterior one (MP_3 and MP_4 forking into MP_{4a} and MP_{4b}) is present in Brixini Emeljanov, 2002, Brixidiini Emeljanov, 2002 and some Mnemosynini (Emeljanov 2002) (Fig. 9C). Oecleini have the anterior trifide type (Fig. 9D). Often, individual asymmetrical variations occur in specimens, but this general pattern is more typical. In several genera, this pattern is often modified with a single posterior MP_{3+4} branch. Such a conformation is found in the following species:

Oecleus mackaspringi Myrie et al., 2019 (Myrie et al. 2019: Fig. 4 with MP_{3+4} misidentified as M_{2+3} and CuA_1 and CuA_2 misidentified as MP_4 and CuA)

Myxia hernandezi Bahder & Bartlett, 2021 (Zumbado Echavarría et al. 2021: Fig. 3 with MP_{1a} , MP_{1b} , MP_2 and MP_{3+4} respectively misidentified with MP_1 , MP_2 , MP_3 , MP_4)

Myxia belinda Bahder et al., 2019 (Bahder et al. 2019) and *M. baynardi* Barrantes Barrantes et al. 2021B) (Barrantes Barrantes et al. 2021a) with Fig. 6 in both papers with MP_{1a} , MP_{1b} , MP_2 , MP_{3+4} , CuA_1+CuA_2 and icu respectively misidentified with MP_1 , MP_2 , MP_3 , MP_4 , CuA_1 and CuA_2 .

In other oecleinian taxa a normal pattern is observed in *Haplaxius dougwalschi* Bahder et al., 2020, (Bahder et al. 2020) and *H. pococo* Bahder & Bartlett, 2021 (Barrantes Barrantes et al. 2021b), in the genus *Bothriocera* Burmeister, 1835, the amber fossil *Bothriobaltia* Szwedo, 2002 (Szwedo, 2002), *Oecleus* Stål, 1862 (Ball and Klingenberg 1935) and *Borbonomyndus* Attié, Bourgoïn & Bonfils, 2002 (Attié et al. 2002). In

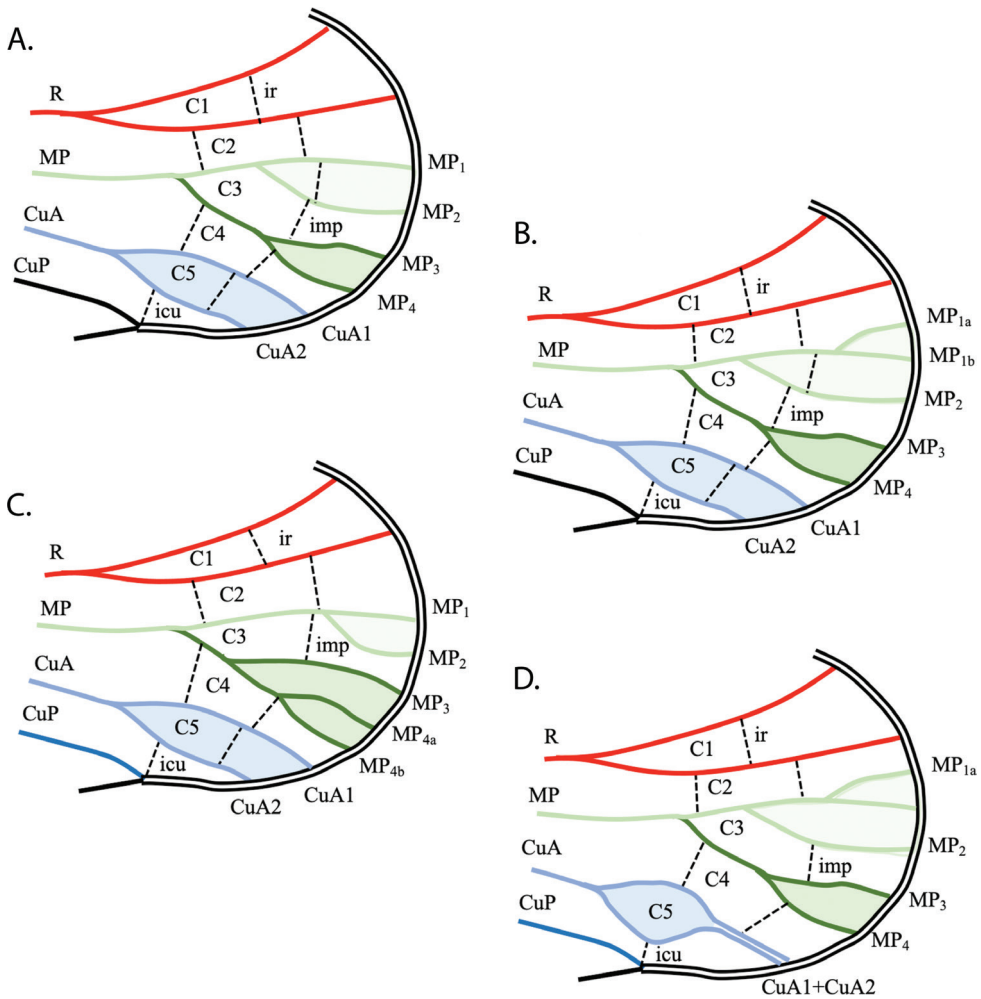


Figure 9. General patterns of MP and CuA veins in planthopper tegmina from (A) ground plan (according to Shcherbakov, 1996; Bourgoïn et al. 2015), of the probable cixiid plesiomorphic condition (B), and with inverted patterns of anterior and posterior MP branches (C) in Mnemosynini and Pintaliini, and Oecleini patterns (D) with CuA branches fused (in many Oecleini MP3 and MP4 are also fused into a single MP₃₊₄ terminal branch).

Meenocixius Attié, Bourgoïn & Bonfils, 2002 (Attié et al. 2002) an unusual late forkings of MP₁₊₂ and MP₃₊₄ at the submarginal line in a 4-terminal MP is observed.

Most oecleine genera exhibit a short diamond shaped C5, distally closed in a single stem of CuA₁+CuA₂ fused in a short stem after the nodal line, or totally fused in a single terminal (Fig. 9D). In *Borbonomyndus* (Attié et al. 2002) and *Bothriocera* (Emeljanov 2002), CuA₁ and CuA₂ remains separated with a probable plesiomorphic elongated C5. These various patterns may be of specific or even generic value, but don't seem stabilized at higher taxonomic rank.

Hindwing venation

Emeljanov (2002), addressed the anastomosis of MP+ CuA in the hind wings of Cixiidae. Four types of connections between the two veins can be described (Fig. 10). The probable most plesiomorphic type or U-type (Fig. 10) shows the two veins still connected by a short mp-cua transverse veinlet as in *Borysthene*s (Emeljanov 2002, fig.6b). The punctate anastomosis between MP₃₊₄ and CuA₁ or V-type (Fig. 10) is found in *Andes* Stål, 1866 or *Pentastiridius* Kirschbaum, 1868, (Emeljanov 2002, fig. 5b, 6b), but a partial fusion of these two veins, or Y-type, seems to be more widely expressed in Cixiidae. The ultimate stage is the complete fusion of MP₃₊₄ and CuA₁ (Fig. 10 I-type) found in several genera of various tribes: *Myndus* Stål, 1862, *Duilius* Stål, 1858, *Cajeta* Stål, 1866, *Pintalia* Stål, 1862, *Eucarpia* Walker, 1857, ... (Emeljanov 2002). While a trend to the fusion of MP₃₊₄ with CuA₁ appears to be quite general in the family, it remains to be described in more genera to better appreciate if these different patterns carry further diagnostical or phylogenetical value. As in many other Oecleini, *Coframalaxius* belong to the I-type, together with the absence of forking of the anterior branch of the media.

Wax pore plates and associated sensory pits on abdominal termites VI–VIII in nymphs

Patterns of these tegumentary glands and sensory units were discussed by Emeljanov (1992), who later presented an evolutionary scenario of its transformation within the Cixiidae (Emeljanov 2002). He posited a five step morpholine with six types arising from a plesiomorphic type with the sensory pits anterior to the wax plates then becoming surrounded by the wax plates, then ultimately reduced to only one sensory pit surrounded by two smaller plates (Emeljanov 2002, fig.10). *Coframalaxius* is a good example of the intermediate type hypothesized by Emeljanov (2002): five sensory pits placed in the anterior row. However, another scenario where the *Coframalaxius* type, quite similar to the pentastirine one, could represent the plesiomorphic condition. It evolved on one side to an apomorphic condition for higher Oecleini taxa with the sensory pits migrated anteriorly to the wax plates (anteromarginal row in *Haplaxius* Fowler, 1904) and on the other side to the multiplication of the lateral sensory pits (Pentastirini) or the progressive fusion of the lateral wax plates with reduction of the sensory pits in the cixiian lineage. As mentioned by Emeljanov (2002) the absence of better knowledge of nymphs of most cixiid tribes is distressing.

Metatibial spines

Two minutes lateral tibial spines are typical of cixiid early nymphs as in *Coframalaxius* (Fig 6D). These spines were present in adult Cixiidae in various numbers, and their absence in adults is interpreted as apomorphic by Emeljanov (2000, 2002) according to his hind leg “disarmament evolutionary scenario”. They are absent in adult oecleines

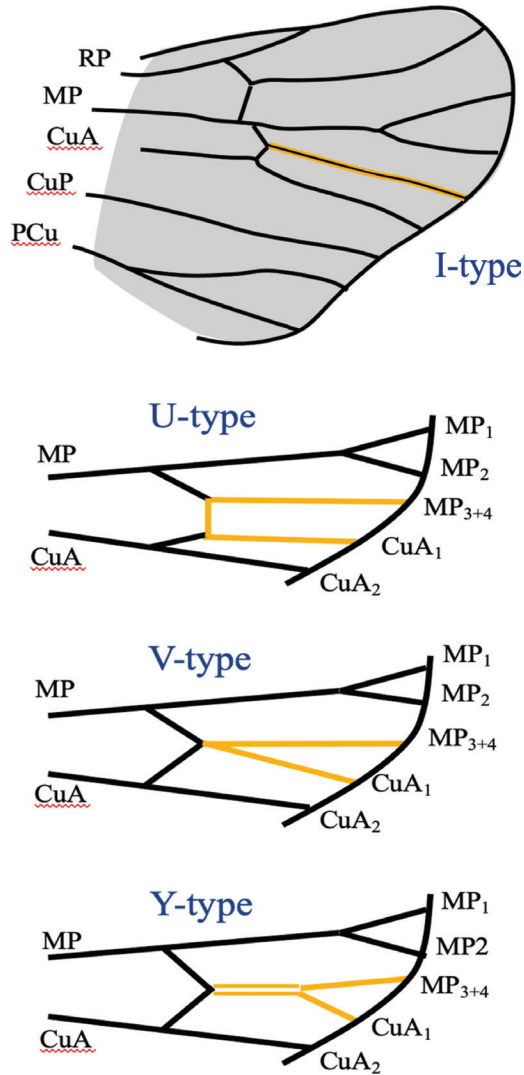


Figure 10. Types of connections between MP and CuA veins in planthopper hindwings. General conformation and respective U-, V-, Y- and I-type observed in Cixiidae.

with the exception in the cave genus *Confuga* Fennah, 1975, in which six minute lateral spines are found (Santos et al. 2019: fig. 8). This condition might be linked to the cave-living adaptation of this species by retention of a nymphal condition in the adult. Lateral spines are present in the other adult cixiids (Emeljanov 2002) - with a few exceptions such as their absence in *Pentastiridius* (*Podaplus*) Emeljanov, 1995, and together with presence of a diastema as in the Uzbekistan species *Pentastiridius* (*Podaplus*) *subterraneus* Emeljanov, 1995). The diastema seems characteristic of the oecleline lineage tribes (Emeljanov 2002).

3. Ecological classification

In the cave, specimens (adults and nymphs) were found in two different “cixiid nests” made of aggregated rootlets by abundant waxy filaments (Fig 1) and forming distinct galleries, in which the specimens were walking. These nests were located in the transitional but obscure zone of the cave at about 1 m from the cave floor for the first one. Several other smaller waxy nests were observed in other root masses, but no adults were observed there. All adults remained inside the nest and were never observed isolated outside the nest. When disturbed, adults would slowly walk away, and were never observed jumping or even flying. In several places in the deep cave zone, nymphs (at least 3rd to 5th instar) were observed walking on the rock surface of the cave walls, usually close to the floor and often close to small roots.

The cave is located in an area well studied with regard to its epigeal fauna and which has been regularly sampled over many years, however no epigeal population had ever been reported. Like all cixiids, the nymphs live in a hypogeous environment, but the adults also stay underground - without being forced to do so by the ants who would exploit them for trophobiosis (Myers 1929; Bourgoïn et al. in prep). The adults live together in the “cixidian nest” consisting of rootlets and waxy filaments produced by the nymphs of different stages which are present along the adults. In the nest, specimens can most likely communicate via the root substrate as do other planthoppers (Hoch and Howarth 1993; Soulier-Perkins et al. 2007), feed and reproduce there. Thus, *Coframalaxius* remains only known from the hypogean habitat where it completes its full life cycle. Based on our observations, rather than a subtroglophile species living temporally or cyclically in hypogean conditions such as its sister taxa *Trigonocranus emmeae* (Hoch et al. 2013), *Coframalaxius bletteryi* should be considered as an eutroglophile species (Sket 2008: 1560). Further field investigations on the life history of *C. bletteryi* will confirm its ecological status.

4. Conservation

Coframalaxius bletteryi, having been found in a single cave in southern France, shows an extreme degree of endemism. The species is specialized to live underground presumably feeding on roots of epigeal plants, and according to field observations, has a presumed small population size. These criteria comply with the IUCN Red Data Book categories “vulnerable”, or even “endangered” (IUCN 2019). This categorization, however, must be regarded preliminary. It is conceivable that the cave where the only known specimens have been found, is but a window to a much more extended superficial underground compartment, or MSS (*milieu souterrain superficiel*, as described by Juberthie et al. 1980), which is well developed in the mountainous regions of Europe (Juberthie 1995). According to Juberthie (1995: 20) the MSS, especially in the Mediterranean regions, at lower elevations forms a mosaic of habitats.

It is thus likely that *C. bletteryi* has a wider distribution in southern France. Nonetheless, there are potential threats to the species and to its environment. Increased

publicity of the occurrence of the new species may increase the number of visitors and put the population of *C. bletteryi* at risk, either through collecting or vandalism, such as damaging roots by trampling or voluntary destruction. Indirect, yet no less severe threats may come from threats to the surface environment, such as droughts, forest fires, as well as deforestation, road construction, and other alteration of surface vegetation. Extirpation of the population in the respective cave would perhaps not mean extinction of the species, however, it would destroy the unique chance to investigate the biology of one of France's rarest endemic species.

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Divergent evolutionary pathways for aggression and territoriality in *Astyanax* cavefish

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Abstract

The surface morph of the Mexican tetra fish (*Astyanax mexicanus*) exhibits strong territoriality behavior and high levels of aggression. In contrast, the eyeless cave-adapted morph from Sierra de El Abra, México, rarely are aggressive and have totally lost the territorial behavior. These behaviors are part of what has been called the cavefish behavioral syndrome. Here, we report that several *Astyanax* cave populations of Sierra de Guatemala, unlike those reported for the Sierra de El Abra cave populations, display significant territoriality and aggression when confined into a reduced space. We discuss divergent evolutionary trajectories in terms of agonistic behavior for cavefish populations inhabiting different mountain ranges.

Keywords

Abra, behavior, stygobyte, troglobite

* These authors contributed equally to this work.

Introduction

Organisms in which variation in behavior can be linked to the environment and their evolutionary history are key to understanding how behavior evolve. The Mexican tetra *Astyanax mexicanus* has both an eyed surface morph and a blind cave-adapted morph. The cave-dwelling *Astyanax* are characterized by conspicuous morphological traits that have evolved in response to their subterranean environment. Among these traits, the most obvious are degenerate eyes and reduced pigmentation (Jeffery 2001; Krishnan et al. 2017). Behavioral differences are also found between the morphs, such as a reduction of intraspecific aggression in some cave-adapted *Astyanax* (Burchards et al. 1985; Parzefall 1985).

Astyanax genus has emerged as a powerful model system for genotype-phenotype analysis because surface fish and cavefish morphs are interfertile and high-quality genome information is available (Gross 2012; Warren et al. 2021). Furthermore, the molecular basis of repeated trait evolution can be examined in this species, as multiple cave populations have independently evolved the same traits (Jeffery 2001; Jeffery 2009). Thirty-four caves are currently known to be inhabited by *Astyanax* (Espinasa et al. 2020). They are found in three mountain ranges in Northern México (Sierra de Guatemala, Sierra de El Abra, and Micos), and one mountain range in Southern México (Guerrero; Granadas and La Joya). Multiple authors (e.g. Bradic et al. 2013; Gross 2012; Herman et al. 2018) have suggested that each mountain range has independent evolutionary histories, although colonization independence has been disputed for the Sierra de Guatemala and Sierra de El Abra by Espinasa et al. (2014). All cave localities (Molino, Caballo Moro, Jineo, Vásquez, and Escondido) involved in this study come from Sierra de Guatemala, with the exception of the Pachón and Tinaja cave populations, which are from Sierra de El Abra (Fig. 1).

Burchards et al. (1985) and Parzefall (1985) showed that epigeal specimens are intensely aggressive and develop territoriality in the laboratory. On the contrary, in the Sierra de El Abra cave populations, aggressive behavior is highly reduced, biting seldom occurs, and fish have lost territorial behavior (Langecker et al. 1995). Even the few attacks performed by cavefish during the behavioral tests have been linked to food-seeking behavior (Elipot et al. 2013). In the natural hybrid populations of Micos and Guerrero, aggression levels are also reduced (Espinasa et al. 2001). The virtual absence of aggressive behavior in these cavefishes has a genetic basis. F1 and F2 hybrids resulting from crosses between epigeal and Sierra de El Abra cave specimens exhibit a very high to intermediate level of aggression, respectively (Parzefall and Hausberg 2001; Elipot et al. 2013). In backcrosses with the cave morph, the aggressive behavior disappears (Wilkens 1988). Of note, F1 hybrids from crosses between Molino cavefish (Sierra de Guatemala) with Surface fish are exceptionally aggressive (Elipot et al. 2013).

Breder (1943) first described the aggressive behavior characteristic of surface Mexican tetra fish as “erratic viciousness”. Among the observable agonistic behavioral patterns are “fin spreading”, “snake-swimming”, and “tail striking”, with the most prominent being “ramming” and “biting” (Wilkens 1988). Subordinate individuals with no opportunity to hide or escape may eventually be killed. The intensity of the aggression depends

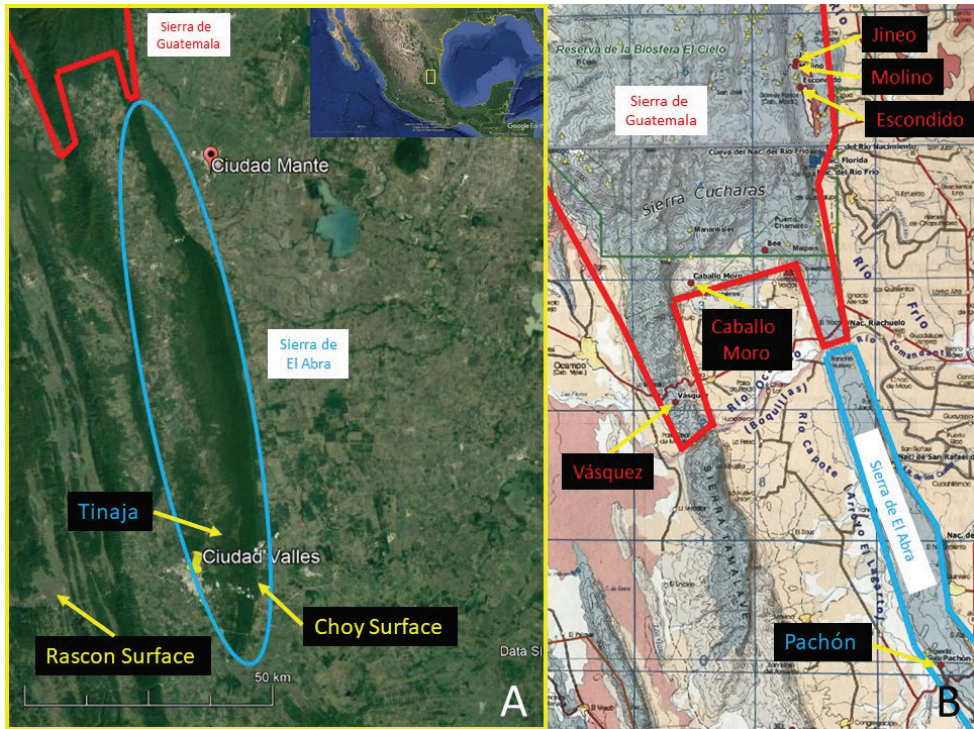


Figure 1. Localities of cave populations **A** satellite photo of Sierra de Guatemala (Red) and Sierra de El Abra (Blue). Inset shows the area of México. Map data 2021 (C) Google **B** topographic map modified from Elliot (2018). Sierra de Guatemala caves are shown in red while Sierra de El Abra caves in blue

on many factors, particularly an individual's size (John 1964), the amount of space available (John 1964), and the overall state of nutrition (Wilkins 1988; Elipot et al. 2013). Vision is not essential to trigger or release aggressive behavior (Espinasa et al. 2005). The agonistic behavior establishes a hierarchical order among the fish (Elipot et al. 2013).

Aggressive interactions and territoriality are correlated, with fish defending an individual space. *Astyanax* surface fish show territoriality that is dependent upon the aquarium space available. In small tanks less than 250 L, surface fish stop schooling and start defending territories (Burchards et al. 1985; Parzefall 2000). Hierarchy and territoriality are linked, with the dominant and most aggressive fish defending the largest area of the tank (Burchards et al. 1985). However, no territoriality behavior has been reported in the cave populations (Parzefall 1983).

The intense dominance-related aggressiveness in surface *Astyanax* is inversely correlated with the serotonin (5HT) levels in the hindbrain raphe nucleus (Elipot et al. 2013; Rétaux and Elipot 2013). A mutation has been identified in the monoamine oxidase (MAO) coding sequence of several cave populations from Sierra de El Abra (Elipot et al. 2014). The P106L MAO mutation would appear as an ideal candidate to serve as the genetic basis of reduced aggression. However, experiments using surface

fish and Pachón cavefish lines with or without the P106L *MAO* mutation have demonstrated that even though *MAO* P106L does increase 5-HT levels in fish, it does not affect aggression and cannot be considered as the genetic basis for the loss of aggressive behavior in cavefish (Pierre et al. 2020). The P106L mutation is present in Sierra de El Abra cavefish but absent in Sierra de Guatemala (Molino, Caballo Moro, Jineo, and Escondido cavefish) as well as in surface fish (Pierre et al. 2020).

Since populations in three mountain ranges (El Abra, Micos, and Guerrero) have independently converged in a reduction of aggression and territoriality, it has been suggested that the loss may be an adaptive change for cave *Astyanax* (Hinaux et al. 2015). The purpose of this study is to resolve whether troglomorphic *Astyanax* can retain aggression and territoriality by studying the Sierra de Guatemala cave populations.

Methods

Cavefish and surface fish localities used in this study can be seen in figure 1. Quantitative data was gathered from fish within four days after being collected from the field in 2016 (Pachón cave collected on August 6th, Caballo Moro cave on May 18th; and Rascón surface fish on August 5th). Data was also gathered from Molino, Vásquez, Caballo Moro, Jineo, and Escondido cavefish, kept in P. Ornelas' laboratory at IBUNAM, México, which had been collected on February 2016, four months prior to the study. Choy surface river and Tinaja cavefish originally bred in Borowsky's laboratory and now kept in Espinasa's laboratory were also analyzed. Fish kept in the laboratory have been under standard husbandry with a water filtration and aeration system. Fish have been fed about 3% of the fish's bodyweight daily. Fish used in experiments were not fed on the 24 hrs before the experiments. Since the Caballo Moro cave holds a mixed population of eyed and eyeless fish, only completely eyeless Caballo Moro specimens were used (Fig. 2). All other cave populations studied only have eyeless fish. Collecting permit # SGPA/DGVS/02438/16 from Secretaría del Medio Ambiente y Recursos Naturales, México, was issued to Patricia Ornelas García. To unify criteria of what constitutes an attack, one of us (LE) went to Rétaux (SR) laboratory to personally observe the pattern of behavior of the specimens described by Elipot et al. (2013) and kept at the French research facility. Afterwards both LE and SR observed the fish kept at Ornelas' Laboratory in México. All counts were performed by at least two observers at the same time to minimize bias. Counts were always very similar and were averaged to present the data.

Aggression

Ramming and biting attempts were counted as attacks following Espinasa et al. (2005). Fish were in a 5-gallon fish tank under two 100-watt light bulbs placed 2 m above the center of the tank for illumination, as described in Espinasa et al. (2001). Fish had an

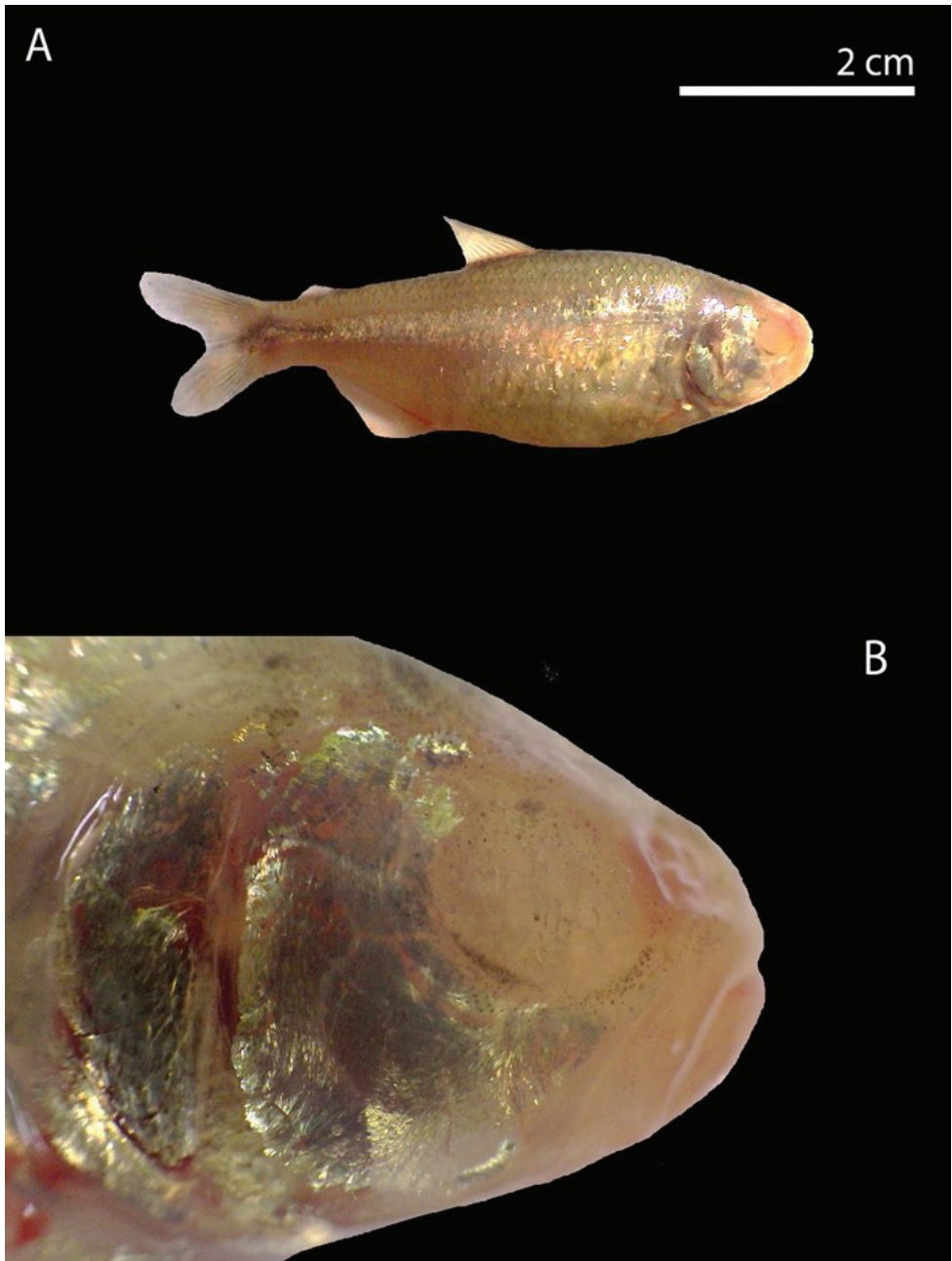


Figure 2. Eyeless Caballo Moro specimen used for aggression and territoriality studies **A** body **B** head. While the Caballo Moro cave is inhabited by both the eyed and the eyeless morphs, only eyeless specimens were used in this study. The other populations used in this study; Molino, Jineo, Vásquez, Escondido, Pachón and Tinaja caves are inhabited exclusively by eyeless fish.

average size of 52.1 mm (max = 62.5 mm, min 43.6 mm). Two assays were used to assess aggression:

1. Four specimens were deposited into a fish tank and left to acclimate for at least five minutes or until two ramming or biting attempts (attacks) were initiated. Afterwards, the number of attacks were counted over a five min period. Replicates with different combinations of individual fish were performed for Molino (n = 18 tests), Caballo Moro (n = 17), Vásquez (n = 9), Jineo (n = 1), Escondido (n = 7), Pachón (n = 6) and Tinaja (n = 6) caves and for Choy (n = 10) and Rascón (n = 6) surface fish. A non-parametric Mann-Whitney U test was used to determine statistical differences among populations.

2. The same procedure was done, but with only two individuals per tank and for the following number of replicates: Molino (n = 9 tests), Caballo Moro (n = 2), Escondido (n = 7), Pachón (n = 8) and Tinaja (n = 6) caves as well as Choy (n = 10) and Rascón (n = 5) surface fish. A non-parametric Mann-Whitney U test was used to determine statistical differences among populations.

Territoriality

To study territoriality, four fish were left in a tank to acclimate overnight. The next morning, they were filmed with a DCRSR42 Sony Digital camera from above for three minutes. Since fish tend to scatter beyond their territories for a few moments after an attack, disrupting territorial patterns, the one-minute section of the film with fewer attacks was selected and examined in the laboratory to track the paths of each individual. For quantification, in the video the tank was divided into four quadrants. The track generated by the fish was analyzed and the number of quadrants not occupied throughout one minute by the individual fish were counted. The assumption was that territorial fish would stay in their territory and some quadrants would not be included in their paths, while non-territorial fish would swim throughout the tank. Samples analyzed were from Sierra de El Abra (Pachón n = 8 tests) and Sierra de Guatemala (Caballo Morro n = 8 and Molino n = 4). A Mann-Whitney U test was done to establish if the populations differed in how many quadrants were not occupied. Fish from other caves (Vásquez, Jineo, Escondido and Tinaja) were also observed qualitatively for signs of territoriality while in their host tanks at Ornelas' laboratory, where they had been acclimatized for months.

Since territoriality in *Astyanax* cavefish has not been previously reported, most authors have never witnessed this behavior in action. For illustration purposes and to facilitate uniformity of criteria in future studies, a video was recorded of cavefish being aggressive and how they establish territories using the intruder essay. Two Caballo Moro specimens were placed into one tank and five into another tank to acclimate overnight. The next morning, they were filmed as described above. Afterwards, an "intruder" fish from the tank with five fish was transferred to the tank with the two fish and left for 20 min to acclimatize, after which, another three minutes were filmed. Again, the one-minute section of the film with fewer attacks was selected to track and mark the preferred territories of each fish. Afterwards, another intruder was added to obtain the swimming paths of four fish.

Results

Aggression

In the four fish assay (Fig. 3A), Sierra de El Abra (Pachón \bar{x} = 0.8 attacks \pm 0.9 SD n = 6 tests with different combinations of fish; Tinaja \bar{x} = 0.5 attacks \pm 0.5 SD n = 6) and Escondido (\bar{x} = 0.5 attacks \pm 0.7 SD n = 7) caves showed significantly lower levels of aggression (P = 0.005–0.0007 Mann-Whitney U test) than the surface populations of Choy (\bar{x} = 13.5 attacks \pm 11.8 SD n = 10) and Rascón (\bar{x} = 52.1 \pm 34.8, n = 6), in agreement with previous studies.

On the contrary, Sierra de Guatemala cavefish populations (Molino cave fish \bar{x} = 16.0 \pm 11.0 SD, n = 18; Caballo Moro \bar{x} = 25.7 \pm 21.0, n = 17; Vásquez \bar{x} = 9.7 \pm 7.3, n = 9; and Jineo \bar{x} = 30, n = 1) had aggression levels not significantly different from surface Choy population (P = 0.68–0.15), and even Caballo Moro cave population was not significantly different (P = 0.06) from the highly aggressive Rascón surface population. All these Sierra de Guatemala cavefish populations were significantly more aggressive (P = 0.002–0.001) than the Sierra de El Abra and Escondido cavefish.

Similar results were obtained in the two fish assay (Fig. 3B). Pachón (\bar{x} = 1.3 \pm 0.9, n = 8), Tinaja (\bar{x} = 0.5 \pm 0.5, n = 6), and Escondido (\bar{x} = 0.7 \pm 0.7, n = 7) cavefish displayed significantly fewer attacks (P = 0.008–0.002), than Molino cavefish (\bar{x} = 9.3 \pm 7.5, n = 9), Caballo Moro cavefish (\bar{x} = 7.5 \pm 0.7, n = 2), Choy surface fish (\bar{x} = 26.4 \pm 21.4, n = 10), and Rascón surface fish (\bar{x} = 83.2 \pm 123.6, n = 5; P = 0). Molino cavefish were not significantly different (P = 0.08) from Choy surface fish. Aggression levels in our four- and two- fish assays over 5 minutes for Molino, Caballo Moro and Vásquez caves are within range of the number of attacks recorded by Espinasa et al. (2005) for three fish over 10 minutes for Molino cavefish (\bar{x} = 35 \pm 19, n = 6; i.e. about 17.7 in 5 min) and for a different surface fish locality, Río Boquillas (\bar{x} = 41 \pm 25, n = 6; i.e. about 20.5 in 5 min). Both Río Boquillas and Río Choy are surface streams neighboring the Sierra de El Abra and less than 10 km from a cave locality, while Rascón population is over 100 km away (Fig. 1). The Rascón population is isolated from the Choy and Boquillas rivers by the 100 m Tamul waterfall and belongs to a different *Astyanax* lineage, as evidenced by its mitochondrial DNA (Ornelas-García et al. 2008). In the four fish assay, the Rascón surface fish were significantly more aggressive (P = 0.01) than the Choy surface fish.

Examples of aggressive interactions of Sierra de Guatemala cavefish can be seen in <https://www.youtube.com/watch?v=8IW7hgZbWI>.

Territoriality

Sierra de El Abra cavefish (i.e. Pachón and Tinaja) show no tendency to establish territories. Pachón individuals swam throughout the tank, preferentially following the edges, crossing paths with other individuals constantly (Fig. 4A). All Pachón individuals shared and used the entirety of the tank. On the contrary, Caballo Moro (Fig. 4B), and Molino

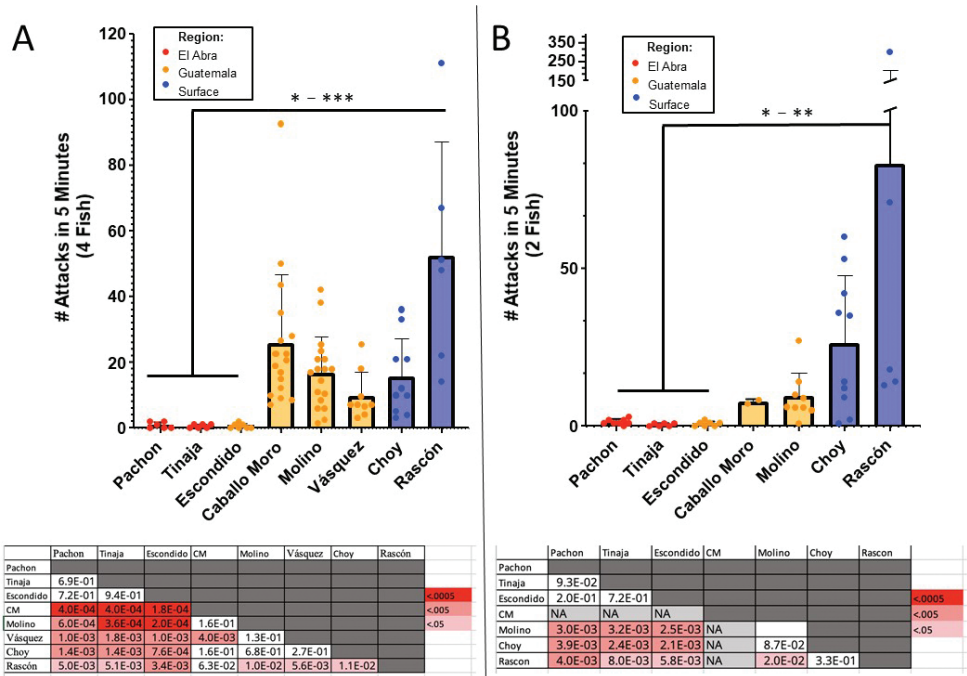


Figure 3. Number (with SD) of ramming and biting attempts in the four fish **A** and two fish **B** assays, for Sierra de El Abra (Pachón and Tinaja), Sierra de Guatemala (Escondido, Caballo Moro, Molino, and Vázquez) cave fish and surface fish (Choy and Rascón). With the exception of Escondido cave, Sierra de Guatemala cave fish were significantly more aggressive than Sierra de El Abra cave fish. In several comparison, Sierra de Guatemala cave populations were as aggressive and not significantly different than some of the surface fish. Tables below graphs show significance levels. Notice that Pachón, Tinaja and Escondido are significantly less aggressive than all of the rest.

fish developed territories where individuals kept to their own areas, with edges of the territories being the places of conflict where aggressive behavior and attacks occurred.

Pachón cavefish had fewer quadrants not traversed in their paths (0.0 +/- 0.0 SD, n = 8 individual fish) in one minute than Caballo Moro (2.5 +/- 0.7, n = 8; P=.00094 Mann-Whitney U test) and Molino (1.25 +/- 1.5, n = 4; sample size too small to test with U test). In all tests, Pachón cavefish shared all sectors of the tank and all individual's paths crossed all four quadrants of the tank. The average time spent in the single most used quadrant for Pachón cavefish was 47.9% (\bar{x} = 28.7 +/- 8.0 seconds out of 60), while Caballo Moro cavefish spent 90.8% (\bar{x} = 54.5 +/- 5.0 seconds out of 60) and Molino 75.4% (\bar{x} = 45.2 +/- 13.8 seconds out of 60) of the time in a single quadrant or corner of the tank and all fish failed to enter between one to three of the other quadrants. This suggests that Sierra de Guatemala cavefish remained within a constrained area or territory, unlike Pachón fish. Fish from other Sierra de Guatemala caves (Vázquez and Jineo) appeared to establish territories at a slower pace than Caballo Moro and Molino fish. Therefore, only a qualitative observation was done in their host

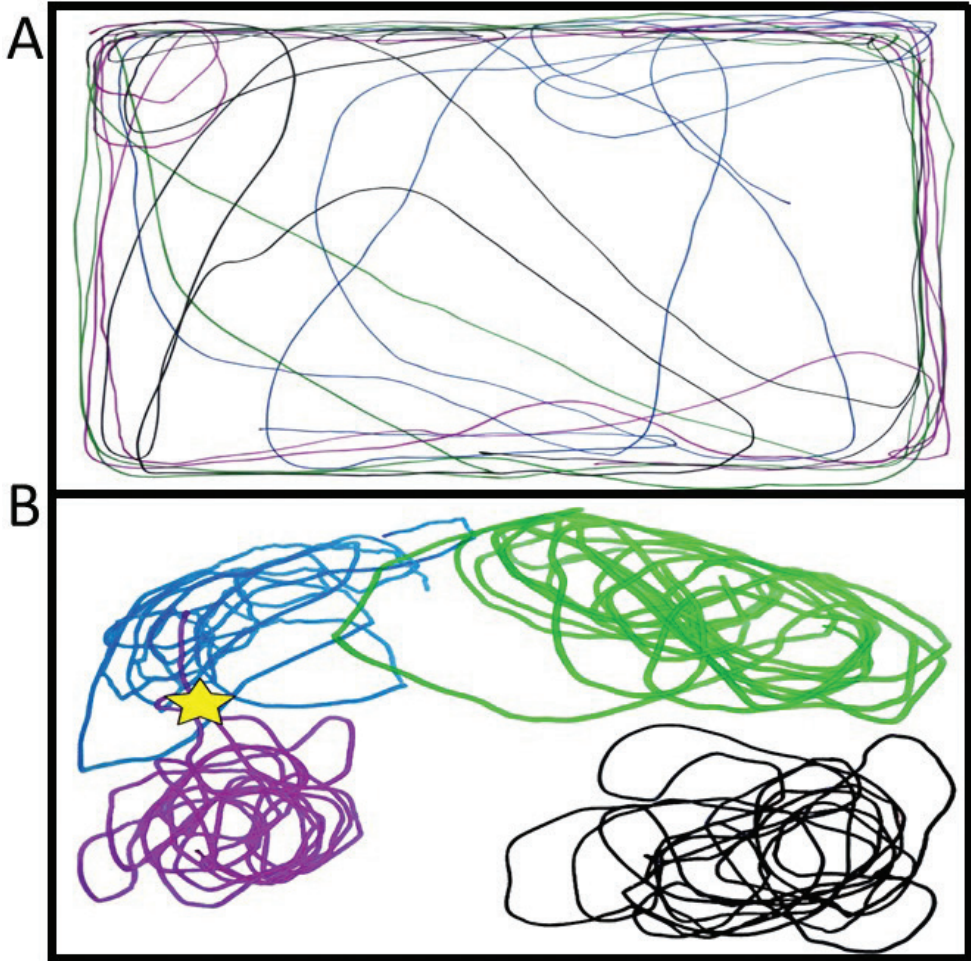


Figure 4. Territoriality from a top view of a tank. Black straight lines represent edges of the tanks (size 20cm X 40cm). Colored lines follow the swimming paths of four individuals over a 1 min period. A Thin lines follow four Pachón cavefish. B Thick lines follow four eyeless Caballo Moro cavefish. Yellow star signals an attack. Specimens used for both cavefish populations are eyeless. Notice that while all Pachón fish swim throughout the entirety of the tank and the paths of individual fish converge over the paths of other individuals, those of Caballo Moro are mostly restricted to a corner, and each individual holds a territory seldom crossed by another individual. However, when they do cross, it can result in an attack.

tanks at Ornelas' laboratory where they had been acclimatized for months for signs of territoriality. In those conditions, cavefish were also seen swimming in paths clearly restricted to only certain areas of the tanks. Qualitative observations of Pachón, Tinaja and Escondido cavefish in their host tanks where they have also been kept for months failed to show equivalent swimming paths restricted to territories.

Examples of territoriality of Sierra de Guatemala cavefish can be seen in <https://www.youtube.com/watch?v=8IW7hgZbWI>.

Discussion

While most authors agree that aggression in *Astyanax* cavefish populations from Sierra de El Abra, Micos, (reviewed in Hinaux et al. 2015) and Guerrero (Espinasa et al. 2001) is highly reduced when compared to surface fish, there have been conflicting accounts for the populations in Sierra de Guatemala. Elipot et al. (2013) reported that both Molino and Pachón fish showed ten times less aggression than surface fish during an hour-long resident-intruder assay. In contrast, an earlier study (Espinasa et al. 2005) reported that Molino cavefish had the “erratic viciousness” equal to surface fish and were clearly more aggressive than the Pachón cavefish.

In the present study, cavefish were directly observed by authors of both the Elipot et al. (2013) and Espinasa et al. (2005) studies to standardize criteria. Five populations were examined (Molino, Caballo Moro, Jineo, Vásquez, and Escondido). Assays used were for two to four individuals. Fish were observed with or without acclimation (like in resident-intruder assays), and with individuals that had just been collected from the field or that had been kept in the laboratory for extended times. In all these tests, 4 out of 5 Sierra de Guatemala cave populations (except Escondido cavefish) have displayed levels of aggression significantly higher than Sierra de El Abra cavefish. The Molino fish have similar histological characteristics of eye reduction to El Abra cavefish (Wilkens 1988). So, their higher levels of aggression are not due to having significantly better vision.

Espinasa et al. (2005) reported that in a “three fish assay”, Molino cavefish had aggression levels over a 10 min period equivalent to surface fish from Río Boquillas. Similar results were obtained by us when comparing against the surface population of Choy. Yet, in our study, surface fish from Rascón were 2 to 5 times more aggressive than both the surface Choy population and the aggressive Sierra de Guatemala cave populations. Two interpretations seem plausible. The first is an ongoing process to loss the aggression, in which most Guatemala fish display “intermediate” aggressiveness between surface Rascón and cave Sierra de El Abra fish. This corresponds to a plastic behavior whose environmental conditions, phylogenetic inertia, or genetic architecture have not promoted the genetic fixation in comparison with El Abra system, while in Escondido the lack of aggressiveness has been favored.

But there is a second alternative that focuses on the variability in aggression levels among surface populations. Sierra de Guatemala cavefish are as aggressive as surface fish from Río Boquillas and Choy, but less aggressive than those from Rascón. Qualitative and quantitative observations in the fish kept at the UNAM laboratory suggest that Rascón fish are more aggressive than other surface populations from streams closer to Sierra de El Abra. While few live specimens are available, tanks with Rascón fish typically need to have reduced number of individuals so as to prevent them from killing each other. Rascón population is isolated from the rest of the surface populations by the 100 m high Tamul waterfall and its known to be genetically distinct by harboring a different mitochondrial haplotype from other surface populations (Ornelas-García et al. 2008) as well as with nuclear data (Herman et al. 2018). So, it could be that Rascón is a particularly aggressive population of surface *Astyanax* and the Sierra de Guatemala cavefish are simply as aggressive as their neighboring surface populations of Choy and Boquillas.

Our study also confirmed that the absence of aggression in the three Molino individuals kept in France used in the Eliot et al. (2013) study was “real” and not an artifact of subjective evaluation or different data collection techniques. We propose that apart from variability among populations, there is variability among individuals within a population regarding their aggression levels. For stochastic reasons, the handful of specimens that were shipped to France displayed little aggression and represented a divergent subset of the natural population with regards to average aggression. In fact, while the norm is that levels of aggression in Sierra de Guatemala cavefish are higher than in Sierra de El Abra, the absence of aggression displayed in the Escondido cavefish housed in Ornelas’ laboratory in México may be the result of this same natural variability. Escondido cave is part of the same hydrologic system as Molino and Jineo Cave (Espinasa and Espinasa 2015). It remains to be seen if for stochastic reasons the pool from which Escondido cavefish were collected happened to have a high number of non-aggressive fish, or if it is the norm throughout that particular cave.

It has been suggested that agonistic behaviors in surface *Astyanax* rely on visual cues and that aggressiveness is no longer performed in darkness at all (Burchards et al. 1985; Parzefall and Hausberg 2001; Langecker et al. 1995). In contradiction, Eliot et al. (2013) reported that surface fish remain aggressive in the dark. These differing findings come about by the fact that Eliot et al. (2013) observed the fish in darkness by using infrared lamps. This method does not consider that many fish (including characids) are able to perceive near-infrared (Meuthen et al. 2012). It is interesting to note that surface fish that have been blinded by surgical lens removal at larval stages are aggressive (Espinasa et al. 2005).

Our analyses of the swimming paths indicate that unlike Sierra de El Abra cavefish, most Sierra de Guatemala cavefish develop territories. Individuals appear to try to minimize contact with each other and the available space is subdivided. The borders of these territories are where most attacks are concentrated. Again, it is worth noting that the cave specimens in these assays were completely eyeless individuals and thus they could not use visual cues to generate a spatial mapping of the tank and their territories. Nonetheless, individuals adjusted their swimming paths to form new and stable territories without the help of visual cues.

Swimming paths of most Sierra de Guatemala cavefish kept inside the laboratory are drastically different from Sierra de El Abra cavefish. Pachón and Tinaja fish typically swim near the edges of the tank, following the entirety of the side and turning at the corners, thus going in circles around the whole tank. Aggressive Sierra de Guatemala fish that hold territories have swimming paths that do not follow the entirety of the side of the tank. Instead, they swim a few strokes and turn around before encountering any physical obstruction. Non-visual spatial mapping must allow them to turn at the frontier of their territories, even when the neighboring individual is distant. At high densities, swimming paths can even be restricted to reduced circling around in areas only slightly larger than the fish itself. Teyke (1985) has shown that active flow sensing, using locomotion and the lateral line are utilized during cavefish navigation. Blind cavefish use a higher frequency of swim cycles than eyed surface fish, consisting of burst and glide in straight trajectories. Santacruz et al. (2015) have suggested that three-dimensional space is learned and remembered differently in *Astyanax* surface fish and cavefish. It is likely

that the modifications to the sensory, processing, and motor neural system of the Sierra de Guatemala cavefish must have occurred such that they are equipped for navigation in perpetual darkness to generate territoriality without the need of visual triggering stimuli.

Based on studies of the Pachón and Micos cave, the surface fish, and F1-hybrids between surface and Pachón fish, Langecker et al. (1995) revealed two releasers for aggressive behavior, ‘natural shape’ and ‘locomotion’. Both would differentially evolve in *A. mexicanus* during the transition from a surface to a subterranean life style. The releaser ‘natural shape’, on the one hand, cannot be manifested in blind cave specimens and became a subject to regressive evolution because of the lack of stabilizing selection. The releaser ‘locomotion’, on the other hand, may be progressively selected because it can still be stimulated by the lateral line system. It is likely to play a role in the new aggressive behavioral patterns of cave fishes (Langecker et al. 1995).

Elipot et al. (2013 and 2014) proposed that the loss of aggressiveness in the Sierra de El Abra cavefish is a “side effect” and not something to be directly selected by living in caves. Raphe serotonergic neurons control aggressive/dominance behavior while at the same time, hypothalamic serotonergic paraventricular neurons regulate food-seeking behavior. They suggested that evolution of the Sierra de El Abra cavefish, in seeking to enhance feeding, lost aggression as a pleiotropic side effect due to modifications of their shared serotonergic neuronal network. We propose that the Sierra de Guatemala cavefish may have achieved the regulation of adaptive feeding behaviors or the control of serotonergic networks through different evolutionary, genetic, and physiologic pathways (e.g., mc4R mutation in Molino cavefish; Aspiras et al. 2015). Thus, the pleiotropic and side effects are different such that aggression and territoriality were maintained.

Espinasa et al. (2021) have found that variability of food resources among different pools within a single cave can be higher than between different caves. Thus, we can assume that it is unlikely that caves in Sierra de Guatemala are environmentally significantly different from those in Sierra de El Abra with regards to selective conditions for loss of aggression and territoriality. We suggest that the behavioral differences are rather due to their independent evolutionary histories, where adaptation occurred through modification(s) of different genes with different pleiotropic effects. Future studies may resolve if aggression and territoriality, or their absence, evolves differently among karstic zones.

Conclusion

Several *Astyanax* cave populations of Sierra de Guatemala, unlike those reported for the Sierra de El Abra cave populations, display significant territoriality and aggression when confined into a reduced space. The Sierra de Guatemala cavefish may have achieved the regulation of adaptive feeding behaviors or the control of serotonergic networks through different evolutionary, genetic, and physiologic pathways than at Sierra de El Abra populations. Thus, the pleiotropic and side effects may be different such that aggression and territoriality were maintained.

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Life history observations of the Illinois state endangered Enigmatic Cavesnail, *Fontigens antroecetes* (Hubricht, 1940) made under simulated cave conditions

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Abstract

The Enigmatic Cavesnail, *Fontigens antroecetes* (Hubricht, 1940), is a cave adapted hydrobioid snail listed as state endangered in Illinois. It is known from only one cave in Illinois, Stemler Cave, and from several caves in the eastern Ozark ecoregion of Missouri. Little is known about the snail's reproductive habits, embryological development, or growth rates. I attempted to gain basic life history information by breeding Enigmatic Cavesnails under simulated cave conditions in the laboratory. Six adult snails were collected from Stemler Cave and held in aerated containers of cave water with one or two cobbles from the cave stream. Containers of snails were housed in incubators set at the average cave water temperature of 13 °C. The snails produced 49 embryos in captivity over the course of 34 weeks. Eggs were deposited singly, attached to the underside of rocks within small pits or crevices. Nearly 82% of embryos developed to hatching. Mean estimated development time of embryos was 70.7 days. Survival of hatchling snails was poor. Limited data available from surviving hatchling snails suggests slow growth rates. The process was replicated with nine Enigmatic Cavesnails collected from Cliff Cave in St. Louis County MO. Captive Cliff Cave snails produced 34 embryos over 46 weeks and varied from the Stemler population in their oviposition behavior, with a majority of eggs deposited on the top surface of rocks. Cliff Cave snail embryos also had longer mean estimated development times (82.17 days).

Keywords

Captive breeding, Fontigentidae, gastropod, stygobiont

Introduction

Subterranean ecosystems are likely the most widespread nonmarine environments on Earth, yet specialized subterranean organisms are among the least documented and studied groups (Mammola et al. 2019). Within stygobitic communities (i.e., restricted to aquatic subterranean habitats), snails are one of the least studied taxa and face elevated risk of extinction due to their limited geographic distribution and habitat degradation, including nutrient enrichment and groundwater contamination (Gladstone et al. 2021). The members of the gastropod clade Hydrobioidea, which includes several families that are collectively referred to as hydrobioids, are extensively adapted to subterranean aquatic habitats (Hershler and Holsinger 1990). These habitats include cave streams, small shallow aquifers that discharge as seep springs (hypotelminorheic habitats), interstitial spaces beneath stream beds (hyporheic zones), and deep aquifers (phreatic zones) (Culver and Pipan 2009; Culver et al. 2012; Alvear et al. 2020). Many hydrobioid species are restricted to springs (crenobionts), living at the interface between subterranean and surface ecosystems (Marenti and Pezzoli 2019). A few species of the North American hydrobioid genus *Fontigens* are found in both cave streams and springs (Steward and Dillon 2004), with surface populations exhibiting full pigmentation and cave-dwelling population showing some features typical of stygobionts, such as reduced pigmentation (Hershler et al. 1990). In one case, a stygobitic hydrobioid thought to be endemic to a single cave was found to inhabit two nearby springs (Delicado 2018). Basic information about life history and the ecological role of most stygosnails is lacking. It is generally assumed that subterranean gastropods feed on detritus (Culver 2005). Simons et al. (2003) used C and N isotope abundance to show that the hydrobioid *Fontigens tartarea* Hubricht, 1963 likely feeds on epilithic microbial biofilms in caves.

The Enigmatic Cavesnail, *Fontigens antroecetes* (Hubricht, 1940) is a minute stygobitic hydrobioid limited to caves in the eastern edge of the Ozarks karst region, USA (Fig. 1). In Missouri, the Enigmatic Cavesnail has been reported from several caves in Perry County, and a single site in St. Louis County, Cliff Cave (Hershler et al. 1990; Wu et al. 1997). The only population known east of the Mississippi River is the type-locality, Stemler Cave in St. Clair County, Illinois (Lewis et al. 2003). *Fontigens antroecetes* has a NatureServe ranking of G2 (Imperiled), is listed as Endangered in Illinois (S1 ranking), and is a species of Conservation Concern in Missouri (S2 ranking). Baseline information on population density and habitat use in Stemler Cave are available (Taylor et al. 2013), however little else is known about the life history of *F. antroecetes*.

High levels of fecal coliforms and other contaminants have been documented in Stemler Cave (Taylor et al. 2000; Dodgen et al. 2017) and the resulting hypoxia has likely led to the extirpation of another stygobiont, the federally endangered Illinois Cave Amphipod (*Gammarus acherondytes*, Hubricht and Mackin 1940) from that cave system (Panno et al. 2006). In a recent review of actions and policies aimed at conservation of subterranean gastropod diversity in North America, Gladstone et al. (2021)

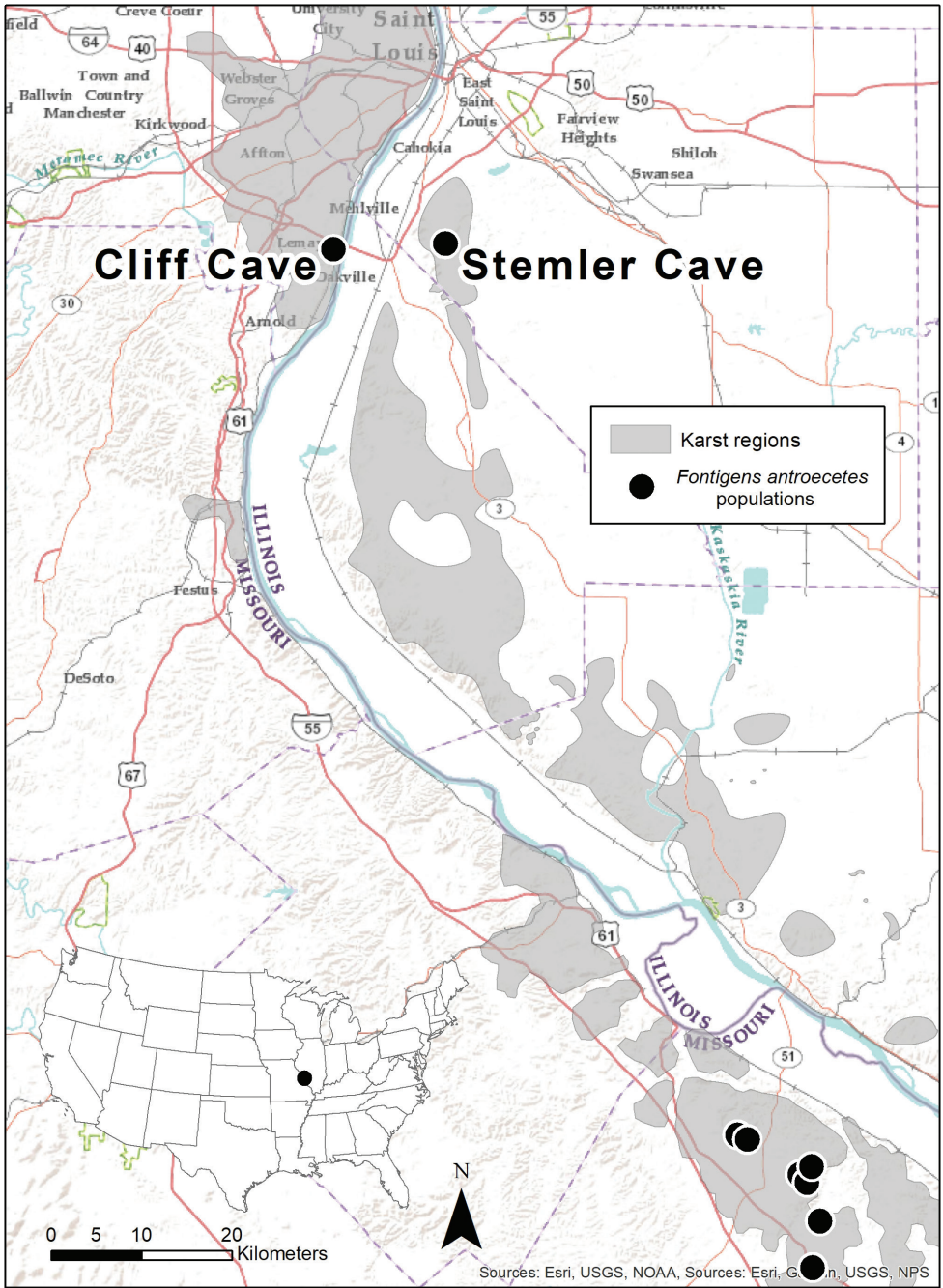


Figure 1. Distribution of the Enigmatic Cavesnail (*Fontigens antroecetes*). Gray areas represent karst regions. Black dots represent known populations.

highlighted the potential usefulness of captive propagating stygobitic snails in gaining information on life history and reproductive biology. Captive propagation could also be an important strategy in reintroduction efforts should isolated populations experience declines or extirpation (Mammola et al. 2022). Here I present life history information for *F. antroecetes* gained through captive breeding under simulated cave conditions, the first such published account for a stygobitic snail.

Methods

Six individuals of *F. antroecetes*, ranging in size from 2 mm to 3 mm shell length, were collected from Stemler Cave, St. Clair County, Illinois USA between 28 August 2016 and 30 May 2017. The snails were variously paired in three different combinations in an attempt to induce reproduction in the laboratory under simulated cave conditions. *Fontigens antroecetes* is gonochoristic and females store sperm once mated. Because externally visible sexual dimorphisms are absent in this species, the sex of individual snails was not determined and it was not known whether any individuals had mated prior to collection.

Cave-like conditions were created within 15 × 10 × 5 cm lidded plastic containers. Each container included cave water and a cobble collected from the stream in Stemler Cave. Cobbles were inspected carefully to exclude any new snails or snail embryos. The cobbles included biofilms on which the snails grazed and substrates for egg deposition. The average cobble size was 12 × 9 × 3 cm. Water depth in the containers was maintained at 3.5 cm. The containers were aerated by aquarium air pumps with a tube fitted through a hole drilled in the lid. The containers were placed in a darkened 46 × 46 × 51 cm electronic cooler set at 13 °C, the mean annual water temperature in Stemler Cave (Taylor et al. 2000). The cooler was fitted with two shelves and could house a total of eight containers, four per shelf.

The experimental containers were removed from the cooler once or twice a week for data collection. The condition of the adult snails was assessed and all surfaces of the cobbles were inspected under a stereozoom microscope for the presence of eggs and to check the condition of developing embryos. The position of any newly deposited eggs was noted (top, side, or bottom surface of the cobble). Water was changed on average every 7.84 days (± 3.3 SD) and cobbles were replaced when biofilms were depleted and to isolate newly detected eggs. Cobbles were replaced on average every 10.26 days (± 6.13 SD). 38.46% of cobble changes were done to isolate newly deposited eggs. Embryonic development times were estimated because embryos were not checked daily to avoid excess handling and potential damage. Mean estimated development times were calculated by averaging the longest and shortest possible intervals between oviposition and hatching, which varied depending on the frequency of observation for each embryo. Newly hatched snails were isolated in separate containers as space in the cooler permitted. Growth rates of a limited number of offspring were estimated by periodically measuring shell length to nearest 0.25 mm with a ruler.

Similar procedures were followed using nine *F. antroecetes* collected from Cliff Cave in St. Louis County, Missouri (USA) on 22 December 2018. The snails were divided

equally between three containers. Water and cobbles from Stemler Cave were added and the containers were placed in simulated cave conditions on 8 January 2019. The null hypothesis that numbers of eggs laid on top, bottom and side of cobbles did not differ between Stemler Cave snails and Cliff Cave snails was evaluated using Fisher's exact test, followed by *post hoc* pairwise Fisher's exact test with Benjamini-Hochberg FDR method for correcting p values in R version 4.1.2 using packages "stats" and "statix" (R Core Team 2001). No regular growth rate data were collected from Cliff Cave offspring.

Results

Stemler cave snails

Wild collected snails from Stemler Cave produced a total of 49 eggs under simulated cave conditions. Eggs were deposited singly, within a clear capsule approximately 1 mm in diameter (Fig. 2). Eggs were invariably deposited within pits, crevices, or depressions on the surface of cobbles. The vast majority of oviposition (93.9%) occurred on the underside of cobbles. Mating and egg laying behaviors were not directly observed during this study.

Hatching success rate was 81.6%. A total of five embryos were accidentally damaged or destroyed during handling for data collection and four embryos failed to fully develop for unknown reasons. Mean estimated embryonic development time was 70.7 days (range 56.5 to 81 days). The uncertainty in development time ranged from 2 to 8 days, with an average of 5.3 days. The hatching process was directly observed in one instance. The snail became more active within the egg capsule in the two days preceding hatching and was observed chewing on the capsule. At the time of hatching the snail crawled out of the capsule through a well-formed hole that it had apparently created. This hatching process has also been documented by Davis (1961) in embryos of the North American freshwater hydrobioid *Amnicola limosa*, Say 1817. Newly hatched *F. antroecetes* individuals had a shell length of approximately 0.75 mm (Fig. 2). Survival of hatchlings was poor, and limited cooler space restricted the opportunity to collect growth rate data on isolated individuals. Data on shell length changes over time from several hatchling snails were combined to produce a growth profile over one year (Fig. 3). These data suggest that captive reared Enigmatic Cavesnails can reach the reported adult shell length of 2.5 mm (Hershler et al. 1990) in approximately 32 weeks.

Timelines and details about egg deposition for each of the three Stemler Cave snail pairings are presented below.

Pair 1 – Two snails collected from Stemler Cave on 28 August 2016 were housed in cave water at ambient room temperature (circa 20 °C) for 142 days, during which 10 eggs were deposited. The snails were transferred to simulated cave conditions on 17 January 2017. Pair 1 snails produced a single egg under simulated cave conditions on 21 February 2017, which failed to develop to hatching. The pair was maintained for

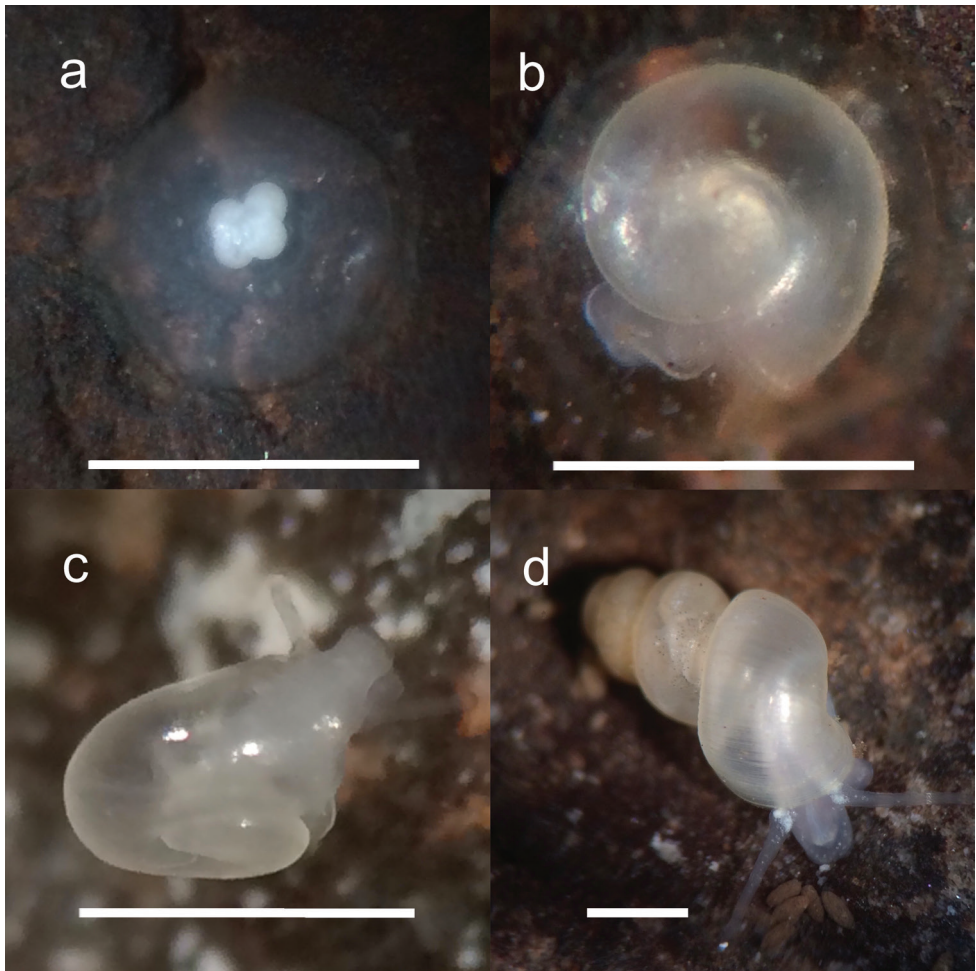


Figure 2. Life stages of *Fontigens antroecetes* observed under simulated cave conditions in the laboratory **a** egg capsule containing two-day old embryo at the four-celled stage **b** well developed embryo at 75 days development **c** newly hatched snail **d** adult lab raised individual. Scale bars represents approximately 1 mm.

total of 70 days until one of the adults was accidentally crushed during data collection on 4 April 2017. The remaining snail was maintained alone under the simulated cave conditions until it was paired with another specimen on 30 May 2017.

Pair 2 – Three snails were collected from Stemler Cave on 4 March 2017 and immediately combined under simulated cave conditions. One snail was found dead on 28 March 2017. The remaining two snails were maintained for 181 days and produced 40 embryos. Egg production appeared to be cyclical during the 25 weeks that the snails were maintained (Fig. 4). Four embryos were damaged due to mishandling and three failed to fully develop. On 1 September 2017 one of the snails was found dead and the remaining individual was then preserved.

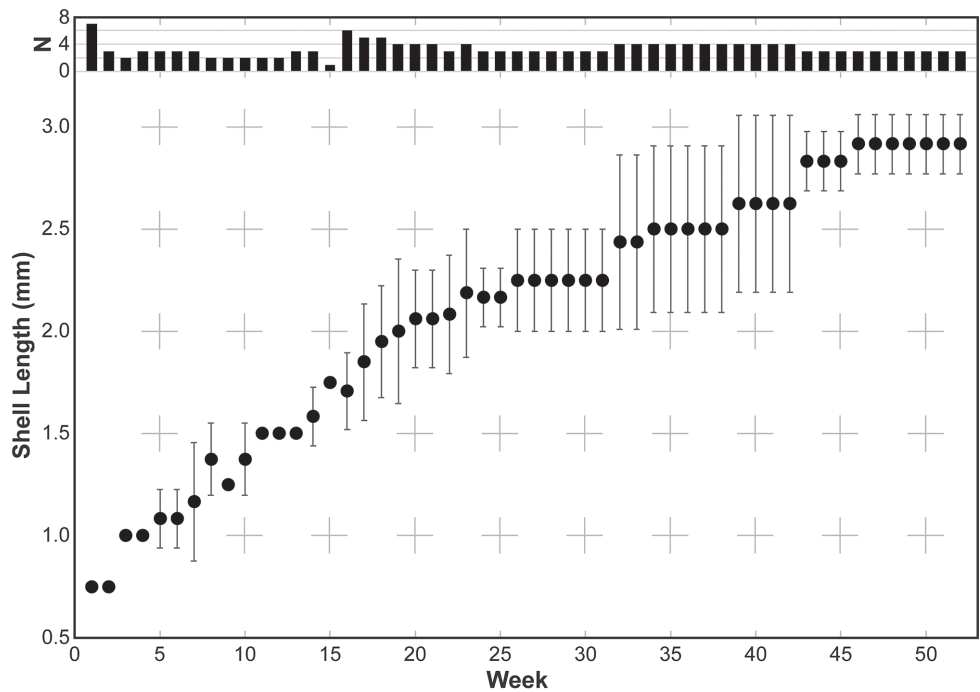


Figure 3. Growth profile of *Fontigens antroecetes* held under simulated cave conditions for up to 52 weeks post hatching. Error bars represent +/- one standard deviation. Histogram indicates number of individuals measured at each week interval.

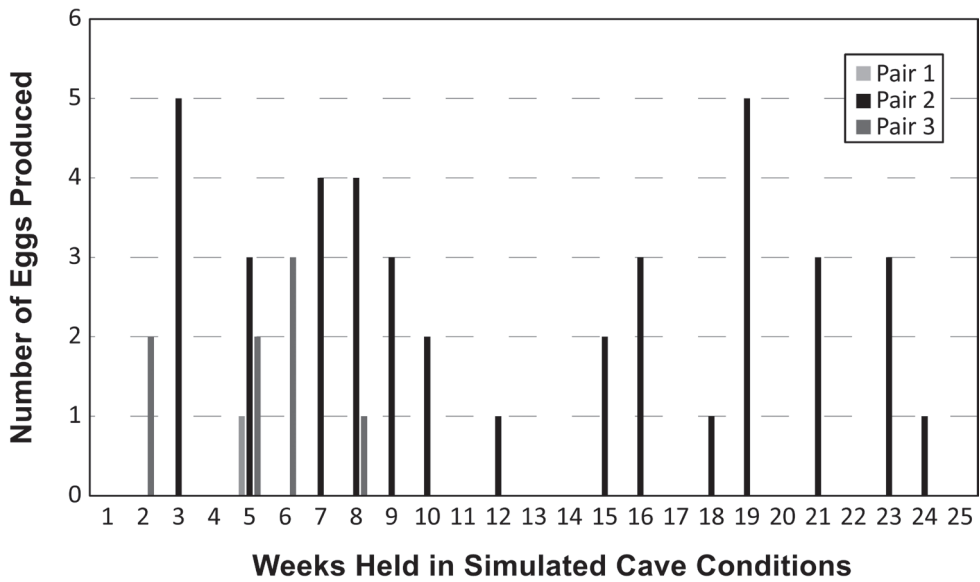


Figure 4. Pattern of egg production by *Fontigens antroecetes* from Stemler Cave, Illinois over 25 weeks in simulated cave conditions.

Pair 3 – A single snail was collected from Stemler Cave on 30 May 2017 and combined with the surviving individual from Pair 1. The pair produced eight embryos during 61 days in simulated cave conditions. All embryos successfully developed to hatching. Both snails were found dead on 31 July 2017.

On 27 June 2019 two laboratory-produced offspring snails, raised in isolation for over two years, were combined in an attempt to yield a second generation of lab-reared offspring. The first egg produced by this pairing was deposited on 10 July 2019, at which time the parents were isolated in an attempt to determine the sex of each snail. A total of 13 eggs were deposited by the female between 10 July and 30 September 2019. Nine embryos successfully hatched (70%). Like the wild collected snails, the female deposited single eggs within in a gelatinous capsule, almost exclusively on the underside of cobbles, often in small pits or crevices. Only one egg was deposited on the top surface of a cobble. The mean estimated development time for the second generation was 73 days. The female, progeny of Pair 1, hatched on 11 December 2016, was 2.5 years old at the time of pairing, and had a shell length of 3 mm. The male, progeny of Pair 2, hatched circa 30 September 2017, was 2.25 years old, and had a shell length of 2.75 mm. The adult female survived until 21 October 2019 and likely died of injuries due to mishandling. The male survived until 22 April 2021. Two sibling second generation lab-reared snails were then reared together. These snails hatched in November 2019 and reached 2.5 mm shell length by the end of November 2020. This pair produced the first viable embryo in September 2021.

Some information about *F. antroecetes* reproduction in Stemler Cave can be gleaned from observations of embryos already attached to rocks collected from the cave stream for potential use in the captive breeding efforts. Well-developed embryos, apparently near hatching, were observed on 14 October 2016, 11 December 2016, 21 June 2017, and 13 November 2021. Early stage embryos were seen on 13 November 2016, 18 December 2017, 29 December 2020, and 13 November 2021. Partly developed embryos were observed on 13 February 2018 and 13 November 2021.

Cliff cave snails

Captive Cliff Cave snails deposited a total of 34 eggs during the course of this study. The first egg was observed on 14 February 2019 and the last on 6 March 2020. All eggs were deposited singly, within an approximately 1 mm wide gelatinous capsule. Unlike the pattern seen in Stemler Cave snails, the Cliff Cave animals mostly utilized pits and crevices on the top and side surfaces of cobbles for oviposition (Fig. 5). Embryonic development time was on average longer (mean estimated time of 82.17 days) and more variable (range 63.5 to 119 days) in the Cliff Cave snails. The uncertainty in development time for Cliff Cave snail embryos ranged from a 2.5 to 14 days day, with an average of 6.5 days. Hatching success rate was 90%. Hatchling shell length was approximately 0.75 mm.

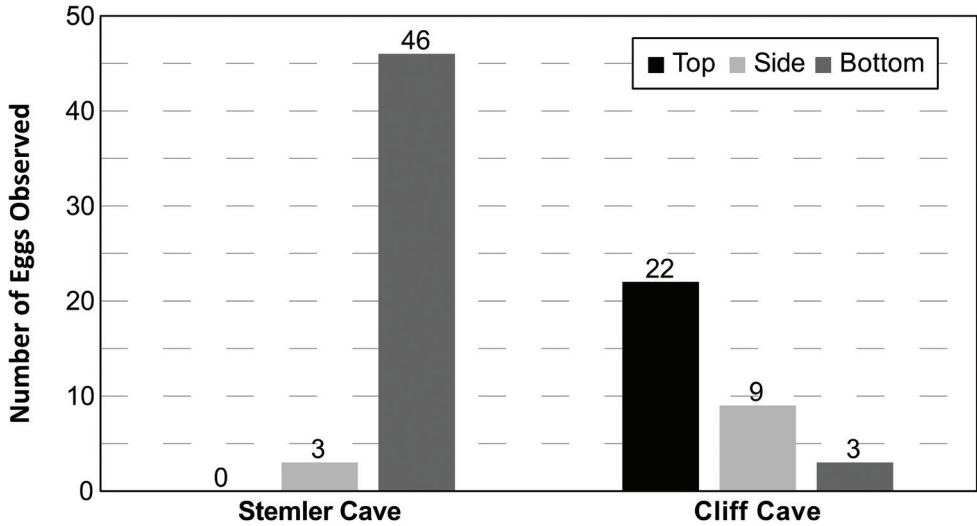


Figure 5. Comparison of egg deposition on top, side, and bottom surface of cobbles in captive *Fontigens antroecetes* from Stemler Cave, IL and Cliff Cave, MO under simulated cave conditions.

Timelines and details about egg deposition for each of the three Cliff Cave snail combinations are presented below.

Container 1 – The three snails in container 1 produced no eggs after 225 days in captivity when all were preserved on 4 August 2019.

Container 2 – A total of six eggs were produced with the first egg observed 14 February 2019, 37 days after the snails were placed in simulated cave conditions. One snail was found dead on 2 May 2019. One additional egg was deposited beyond that date. A second snail was accidentally crushed during handling on 27 June 2019. No additional eggs were deposited beyond that date, and the remaining snail was preserved on 4 August 2019.

Container 3 – The first egg was discovered on 18 April 2019, 100 days after the snails were placed in simulated cave conditions. Sixteen additional eggs were deposited between 18 April and 16 July 2019. At that time, each of the three snails were sequentially isolated in an attempt to gain information about egg production by individuals. The first snail isolated produced no eggs before it was preserved on 6 August 2019. The two remaining snails were left paired between 16 July and 30 July 2019, during which four additional eggs were produced. These two individuals remain alive at this time, over 1000 days after entering simulated cave conditions. One produced a single additional egg on 18 August 2019. The remaining snail produced six additional eggs, with the last egg deposited on 6 March 2020.

A few Cliff Cave offspring were successfully raised to maturity. Three individuals hatched in September 2019 had reached 2.75 mm shell length by July 2020 and began

producing a second generation in the lab by late August/early September 2020. Twenty-six eggs were observed between September 2020 and September 2021. Oviposition varied from the wild-collected snails in that a higher percentage of eggs were deposited on the bottom side of cobbles (54%). One surviving individual continued to produce viable embryos in September 2021.

Discussion

Egg production and oviposition

The deposition of single eggs within capsules, attached to substrates appears to be typical for several genera of hydrobioid snails (Johnson et al. 2013). The two by three matrix of oviposition locations on cobbles differed between Stemler Cave and Cliff Cave snails ($p < 0.0000$), with *post hoc* pairwise Fisher's exact tests demonstrating marked differences between caves for both bottom versus side ($n = 61$, adjusted $p < 0.0000$) and bottom versus top ($n = 71$, adjusted $p < 0.0000$) and less pronounced differences between side and top ($n = 34$, adjusted $p = 0.0368$) of cobbles. The differences in oviposition behavior observed between the Stemler Cave and Cliff Cave snails in this study could be explained by differences in the physical conditions in the two caves. Unlike Stemler Cave, the gravel and cobble substrates in Cliff Cave are largely cemented together with calcium carbonate deposits, limiting interstitial spaces and making the undersurfaces of cobbles less accessible to snails. The tendency for Cliff Cave snails to deposit eggs on the upper and side surfaces of cobbles could be a response to the high degree of calcium deposition covering the streambed, limiting access to the undersurface of substrates. Stemler Cave has a large recharge basin (18 Km²) and is subject to intense pulse floods following heavy rains. Oviposition on the protected undersides of substrates could be an adaptation in Stemler Cave snails to intense stream flow.

Almost nothing is known about stygobitic snail reproduction in nature. Slay et al. (2016) reported that *Fontigens* in the Ozarks have been observed depositing eggs in April. My observations of embryos attached to rocks collected from Stemler Cave suggest that reproduction in that population may be on ongoing and year-round. This is in accordance with observations by Bichuette and Trajano (2003), demonstrating that two species of subterranean hydrobioids in the genus *Potamolithus* showed non-seasonal reproduction with a high percentage of mature females present throughout the year.

Growth rates and longevity

Mean estimated embryonic development time of 70.7 days for Stemler snails and 82.17 days for Cliff Cave snails is surprisingly long considering the small size of the embryo, and likely reflect slow growth and lower metabolic rates typical of cave-adapted organisms (Culver and Pipan 2009). I have conducted unpublished studies of reproduction and growth of *Physella acuta* (Draparnaud, 1805) from Stemler Cave in the laboratory

under simulated cave conditions. *Physella acuta* is a snail known for its ability to invade new habitats (Dillon et al. 2006) and only opportunistically inhabits caves. *Physella acuta* had a considerably faster life cycle under simulated cave conditions compared to *F. antroecetes*. Mean embryonic development time was 18.5 days, and snails grew to adult size and began reproducing after approximately 60 days. Weck and Taylor (2016) reported similar patterns with another cave-dwelling population of *Physella* (likely *P. gyrina*) from southwestern Illinois.

No comparable studies of sytobitic snail growth and longevity are available, although a few studies of epigeal hydrobioids have been published. *Potamopyrgus antipodarum* (Gray 1843) reared in aquaria at 12 °C grew at a rate of 0.007 mm/day over 250 days, and had a predicted life span of 16–18 months based on observations of a wild population (Dahl and Winter 1993). In this study, the average growth rate of *Fontigens antroecetes* raised at 13 °C was identical (0.007 mm) over the same period of time (250 days). Lassen and Clark (1979) raised three species of marine hydrobioids in the laboratory. Development time for *Hydrobia* spp. was 12–15 days at 15 °C, the field temperature during peak reproduction in nature. Lab-produced *Ecrobia ventrosa* (Montagu, 1803) offspring reared at 15 °C began producing second generation eggs after 13 weeks. Development times more comparable to those observed in my study (49 to 70 days) were only seen when temperatures were reduced to 4 °C. Some parallels to the trends observed in *F. antroecetes* are also seen the amnicolid hydrobioids living in the cold (3.6 °C) temperatures of Lake Baikal. Ropstorf and Sitnikova (2006) made *in situ* observations that showed year-round reproduction, long development times (several months) and slow growth rates, with up to 4 years to maturity for Baikalian snails.

Brown et al. (2008) considered North American freshwater hydrobioids to be annuals with seasonal recruitment in cold systems. *Amincola limosa* Say 1817 and *Pomatopsis cincinnatiensis* (Lea 1840) both exhibit seasonal reproduction and an annual life cycle (Van der Schalie and Getz 1962; Pinel-Alloul and Magnin 1973). The observations in this study suggest that *F. antroecetes* is not an annual, and follows life history strategies typical of other cave-adapted animals: slow growth, late age at maturation, and long lifespan (Fiser 2019). Enigmatic cavesnails take approximately 10 weeks to complete embryonic development, 32 weeks to reach minimum reported adult size, at least one year to reach sexual maturity, and can survive for at least 3 years in captivity. A life cycle of 2–3 years for this species is likely.

The results obtained in this study provide a possible approach to gaining life history information about snails from other subterranean systems. Dr Paul Johnson (personal communication, 2 December 2021) attempted to develop protocols for captive rearing the federally endangered Tumbling Creek Cavesnail, *Antrobia culveri*, Hubricht, 1971, at the Tennessee Aquarium. The project utilized *Antrorbis breweri*, Hershler & Thompson, 1990, the Manitou Cavesnail from Alabama, and *Fontigens* spp. as surrogates. They were able to propagate some snails, but not in appreciable numbers sufficient for repopulating a portion of a cave. Future studies could explore more effective methods for maintaining laboratory populations of stygobitic snails as a possible refugium to protect against catastrophic events such as chemical spills. These

laboratory populations could then provide a source for reintroduction after the cave stream has sufficiently recovered. This approach has been used for subterranean aquatic species in the Edwards Aquifer of Texas (Britton et al. 2020). These future studies of laboratory culture of *F. antroecetes* should explore refinement of methods for handling of cobbles and specimens to reduce accidental mortalities from handling and improve recruitment of hatchling snails.

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