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RESEARCH ARTICLE



Benthic species assemblages change through a freshwater cavern-type cenote in the Yucatán Peninsula, Mexico

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Abstract

We studied benthic assemblages through X-Batún, a continental freshwater cenote and its associated submerged cave located in San Antonio Mulix (Yucatán, Mexico). Using cave diving techniques, we collected sediment samples at four zones of the system. We extracted and counted individuals of benthic species in three replicates of 5 grams of wet sediment at each site. The biological composition was integrated by 15 species from eight higher taxonomic groups. Non-metric multidimensional scaling distinguished four assemblages that coincided with surface, open water, cavern and cave zones. ANOSIM test revealed significant differences between the assemblages. In the deeper zones of the cenote characterized by twilight and total darkness, Ostracoda and Gastropoda show the highest diversity and abundance, with practical absence in surface sediments. This pattern may suggest ecological interactions with chemosynthetic bacterial activity. Surface shows an assemblage typical of epigean environments. Environmental variables along the cenote varied little from the upper layers to bottom. Linear correlation and detrended canonical analysis revealed that light is the main driver of benthic species assemblages. Temperature, pH, and dissolved oxygen exert higher influence at individual biological benthic assemblage in X-Batún.

Keywords

benthic assemblages, cenote, groundwater, light gradient, Ostracoda, zonation

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Introduction

Yucatán is a karstic platform composed primarily in Cenozoic limestone which was formed mainly from coral reefs (Marín et al. 2000; Pacheco and Alonzo 2003). One of the main characteristics of the hydrogeology of Yucatán is the presence of "cenotes": water-filled dolines formed by the dissolution and collapse of limestone rock (Gaona-Vizcaino et al. 1980). Cenotes are highly heterogenous regarding their geomorphology, given distinct paths of dissolution and diagenesis of carbonate (MacSwiney et al. 2007; Brankovits et al. 2021). Most cenotes are, therefore, unique in shape and structure, with highly variable internal features such as subterranean passages, caves, conduits, and display different degrees of interaction with marine and terrestrial environments. The high availability of ecological niches and habitats within cenotes provides conditions for high levels of endemism and complex ecological interactions for both, terrestrial and aquatic taxa (Calderón-Gutiérrez et al. 2018; Chávez-Solís et al. 2020). In fresh and anchialine environments of the Peninsula, for example, about ~30 stygobiont (obligate aquatic subterranean) endemic malacostracan species have been recorded so far (Angyal et al. 2020).

In subterranean waters, biological composition is known to be primarily structured by environmental gradients (Sánchez et al. 2002; Pellegrini et al. 2018). Bulk organic matter, water quality and sun light are key for species diversity and distributions (Martí et al. 2004; Brankovits et al. 2017; Bishop et al. 2020).

The general view of the aquatic biodiversity structure in subterranean waters suggests that abundance and diversity of organisms progressively decrease from the highly populated and energy-rich well illuminated zones to the energy-deprived and poorly populated deeper zones (Mammola 2019). This assumption is supported by the fact that well-illuminated zones of the cenotes, usually known as "windows to the surface", are the regions where primary production by photosynthetic organisms happens and where allochthonous organic material enter to the subterranean system (MacSwiney et al. 2007; Hendus et al. 2019). However, there is increasing evidence that food sources originated from bacterial communities and nutrient transport are relevant for supporting trophic web chains in zones where sun light is limited or totally lacking (Brankovits et al. 2017; Chávez-Solís et al. 2020). Benthic assemblage structure along subterranean waters can therefore significantly vary from site to site, even within the same region (Rodríguez 2002; Cresswell 2019; Borisov et al. 2021).

Several publications deal with the characterization of the stygobiont fauna that inhabits the water column and the associated microhabitats (such as the surface of rocks or organic material deposits) of the Yucatán cenotes and their caves (eg. Álvarez et al. 2015; Espinasa et al. 2019; Angyal et al. 2020; Liévano-Beltrán and Simoes 2021), but only scarce information is available about the benthic communities of the Yucatán aquifer complex.

Smirnov and Elías-Gutiérrez (2010) investigated the biocenotic characteristics of 25 cenotes, lagoons, and wetlands of the Yucatán Peninsula, which concluded that among the invertebrate remains, ostracodes were the most common, as they were absent only in four of the studied freshwater bodies. Grego et al. (2019) published

new distribution records and new species of freshwater subterranean gastropods from benthic samples of some continental cenotes in the eastern part of Yucatán state and found three species of which two were new to science. Except these efforts, despite the enormous volume of the Yucatán cenote aquifer, no systematic and ecological studies of the cave benthic fauna had been done so far and key questions about benthic communities remain unanswered, such as how benthic fauna are structured along the Yucatán Peninsula tropical subterranean aquifer? what are the ecological interactions established between biotic and abiotic components? and how community is affected at regional scale by anthropogenic activities developed in surface?

In this study, we characterize the benthic assemblages, based on invertebrate and other remains of the cenote X-Batún, and evaluate how these assemblages vary along four well-differentiated zones of the cenote. We collected information about the variation of the physical and chemical parameters of the water column continuously along the cave system, allowing explanation of how the distribution of benthic invertebrates could be determined by environmental characteristics.

Methods

Study area

The study system was cenote X-Batún in San Antonio Mulix, Umán, Yucatán, México (20°40'26.6"N, 89°46'22.1"W) (Fig. 1). This cenote is part of the Ring of Cenotes, a circular fracture zone with high density of sinkholes identified as the outer rim of the crater where a meteorite impacted the peninsula 66 million years ago (Bauer-Gottwein et al. 2011).

Cenote X-Batún is an oligotrophic freshwater cenote open for the tourism, characterized by a fully solar-irradiated open water pool covered with dense vegetation. X-Batún surficial waters are dominated by aquatic rhizomatous herbaceous perennials (Nymphaeaceae). The system is characterized by a water-filled cavern in the twilight zone with fragmented rock slopes covered with decaying organic material, and with two submerged cave passages in the dark zone. In Yucatán Peninsula, three seasons occur: a dry season with high temperatures and low rainfall of about 400 mm yr⁻¹ during March to May, a rainy season with frequent rainfall with values increasing from 900 to 1400 mm yr⁻¹ takes place from June to October, and a cold-fronts season with winter storms and occasional rainfall from November to February (Pérez-Ceballos et al. 2012).

Sampling procedure, specimen sorting and identification

A sampling campaign was undertaken on 25 May of 2021 by Cenoteando team (Universidad Nacional Autónoma de México, UMDI-F. Ciencias-Sisal, https://www.cenoteando.mx/). This campaign was authorized by the Secretaría de Desarrollo Sustentable del Estado de Yucatán, Dirección de Gestión y Conservación de Recursos Naturales with authorization



Figure 1. Location of the studied cenote in the Yucatán Peninsula. Yellow dot illustrates the geographical position of Cenote X-Batún within the Ring of Cenotes.

number VI-0884-2021. Using scuba diving techniques, we collected sediment samples at four zones of the cenote. Three of the four examined zones were defined and adapted from Chávez-Solís et al. (2020), as follows: "water surface" (S) is the uppermost, fully illuminated water layer with photosensitizing partially submerged aquatic vegetation, algal communities and with aquatic fauna typical of surface waters. "Open water" (OW) or "cenote pool" is a region under the water surface that receives direct influence from the surroundings i.e., the air interface, organic debris, interaction with animals from the surface and, in some cases, direct or indirect sun light. The "cavern" (CN) is the transition zone from the open water to the cave: a region where sunlight is still perceivable but has no direct incidence, there is less external influence and has no vertical access to the surface. The "cave" (C) is the aphotic region, with minimum or no influence from external factors and no vertical access to the surface (Table 1, Fig. 2). At each of these four zones, two sediment samples were collected using plastic Ziplock bags. Sediments were taken only from the uppermost three centimeters of the cenote floor to capture only recent biological material (not fossil or subfossil). Final weigh at each bag was about 2 kg of wet sediment.

Specimen extraction and counting were carried out in three replicates of 5 grams of wet sediment at each of the samples collected. We used a stereomicroscope for specimen sorting and those with soft parts, complete shells or well-preserved valves were extracted and counted as individuals. In case of ostracodes, to avoid over representation,

Table 1. Data of the sampling sites and characteristics of sediment samples collected from four zones of cenote X-Batún. DOM = decaying organic matter.

Sample code	Hydro-zones	Illumination	Depth	Characteristics
S1	water surface	Sunlight	<1.0 m	thin layer of sediment composed by algae and DOM
S2	water surface	Sunlight	<1.0 m	thin layer of sediment composed by algae and DOM
OW1	open water	Sunlight	-7.1 m	thick layer of sediment, lot of DOM
OW2	open water	Sunlight	-7.5 m	thick layer of sediment, lot of DOM
CN1	cavern	Twilight	-24.0 m	thick layer of sediment, lot of DOM
CN2	cavern	Twilight	-27.7 m	thinner layer of sediment than at sampling site CN1, less DOM
C1	cave	Darkness	-44.3 m	very thin layer of sediment covered with calcite crystal plates,
				DOM not visible
C2	cave	Darkness	-37.7 m	thicker layer of sediment than at sampling site C1,
				DOM not visible



Figure 2. Schematic drawing of Cenote X-Batún with the four different zones of the cenote evaluated, and sampling sites in the sediment layer. S1 = water surface 1, S2 = water surface 2, OW1 = open water 1, OW2 = open water 2, CN1 = cavern 1, CN2 = cavern 2, C1 = cave 1, C2 = cave 2.

we counted the right valve only, when free valves were recovered. For each zone of the cenote, species average values were calculated. Individuals were deposited in Eppendorf tubes with 96% ethanol and are available in the collection of ostracodes of the Tec-nológico Nacional de México – I. T. Chetumal.

Specimen identifications were carried out with invertebrate specialized literature (e.g. Meisch 2000; Karanovic 2012 for ostracodes; Grego et al. 2019 for gastropods) and if possible, by consulting taxonomists with experience on invertebrate aquatic tropical taxa. Representative individuals of each of the taxonomic groups found in the cenote were photographed with an Olympus BX51 microscope. Using the photo stacking method and the weighted averaging algorithm in Helicon focus 7.6.6 software, we obtained final photographs that show specimen details focused (Fig. 3).

Environmental variables were measured in situ using an YSI Exo 2 Multiparameter probe (made in USA, range 0 to 200 mS/cm, respond T63<2seg, resolution 0.0001 to 0.01 mS/cm, type of sensor nickel cell 4 electrodes). Profiles of temperature, pH, total dissolved solids (TDS), conductivity and dissolved oxygen were obtained. Along the water column, measurements were taken every 2 cm, and more than a thousand values were recorded for each variable from the top to deepest bottom of the cave. The resulting database was manually edited by eliminating those records duplicated by depth. Final dataset consisted in 350 records.

Statistical analysis

Benthic assemblage distribution through cenote X-Batún

We determined ecological attributes of the evaluated zones of the cenote using the following diversity metrics: species richness, evenness and α diversity, using Shannon diversity index. The ANOVA test was used to determine statistical differences in the diversity between the zones of the cenote.

Benthic species associations along the Cenote X-Batún were determined with nonmetric multidimensional scaling (NMDS). The NMDS generates an ordination in a two-dimensional space, representing the pairwise dissimilarity between species according to their occurrences. We first calculated the Bray-Curtis dissimilarity coefficient, using total abundance data, previously transformed by square root (Clarke and Warwick 1994).

Analysis of similarities (ANOSIM) (Clarke 1993) was then used to identify significant differences in composition and abundance of benthic assemblages between the zones defined in the NMDS analysis. To test the statistical contribution (%) of each species to the composition and abundance similarity determined in the Bray-Curtis matrix we used the SIMPER analysis. Statistical analyses were conducted in the software package PRIMER v6 (Clarke and Gorley 2006).

To estimate the relative importance of the environmental variables measured along the cenote associated with the species assemblage structure, we performed a linear correlation test (Pearson correlation).

Detrended Correspondence Analysis (DCA) with detrending by segments and non-linear rescaling was conducted to estimate major gradients in species composition using the CANOCO version 5 package (Šmilauer and Lepš 2014). The abundance data were logarithmically transformed, and rare species were down weighted. Ordination site scores were correlated to medians and coefficient of variation of all



Figure 3. Benthic taxa of sediment samples extracted from Cenote X-Batún A Pseudocandona sp.
B Cypridopsis vidua (Müller, 1776) C Darwinula stevensoni (Brady & Robertson, 1870) D Cyprididae sp.
E, G Arcella megastoma (Penard, 1902) F Centropyxis discoides (Penard, 1890) H–J Pyrgophorus coronatus (L. Pfeiffer, 1840 K Bothriopupa sp. L Tyrphonothrus sp.

measured environmental factors by means of Kendall tau. Medians and coefficients of variation of all environmental variables were plotted onto DCA ordination diagram as supplementary environmental data for ecological interpretations.

Environmental variables were standardized and added by forward selection using the Monte Carlo permutation test with 999 permutations ($\alpha = 0.05$). Calculations and final ordination graphs of the DCA were performed using the software Canoco version 5 (Šmilauer and Lepš 2014).

Results

Species diversity metrics in Cenote X-Batún

A total of 15 aquatic species belonging to eight higher taxonomic groups were found in the sediments of Cenote X-Batún. The main groups identified are foraminifers, testate amoebae, bivalves, gastropods, copepods, ostracodes, mites and insects. We identified the specimens down to genus level and five of them were also identified to species level.

Species richness was relatively homogeneous throughout the cenote with values ranging from seven to eight species at each zone evaluated, but most zones display a unique species composition (Fig. 4). Ostracodes display the highest species richness with four species.

In terms of abundance, ostracodes were also the most abundant group in the cenote. The species *Darwinula stevensoni* displayed the highest abundance with 98 ind g-1, followed by testate amoeba *Arcella megastoma* (68 ind g⁻¹), the ostracod *Cypridopsis vidua* (46 ind g⁻¹) and the gastropod *Pyrgophorus coronatus* (45 ind g⁻¹). Alpha diversity based on Shannon index values range from 1.1 to 1.84, revealing relatively low species diversity in the system. The ANOVA test reveal that there are not significant differences between Shannon diversity index at each zone evaluated (F (3,4) = 3.07, p = 0.15), and therefore, diversity can be considered homogeneous along the cenote. Evenness was highly variable ranging from 0.43 to 0.9, suggesting differences in species total abundances between sites. The open water zones display almost homogeneous abundances between their species, whereas surface and cave zones display, variable abundances between their respective species (Fig. 4).

The benthic assemblages were not continuously distributed along the cenote (Table 2). Ostracodes and gastropods were mostly distributed toward the deepest zones of the cave, which is characterized by less light availability. Chironomids, testate amoeba and copepods were distributed mainly toward the surface.



Figure 4. Benthic fauna diversity metrics along an environmental gradient in cenote X-Batún.

Four different assemblages were discriminated in the NMDS ordination with 70% of Bray-Curtis similarity (Fig. 5). The assemblage G1 coincided with water surface zone and was constituted by testate amoeba, chironomids, mites and copepods. The assemblage G2 was integrated by testate amoeba, gastropods and ostracodes. All from open water zone of the cenote. Assemblages G3 and G4 were constituted by cavern and cave sites, respectively. On these two zones of the cenote, ostracodes and gastropods were the species better represented.

Table 2. Occurrence of the benthic taxa extracted from eight sediment samples of four zones of Cenote X-Batún. S1 = water surface 1, S2 = water surface 2, OW1 = open water 1, OW2 = open water 2, CN1 = cavern 1, CN2 = cavern 2, C1 = cave 1, C2 = cave 2. + = presence, - = absence.

Taxa	Higher taxon	S 1	S 2	OW1	OW2	CN1	CN2	C1	C2
Foraminifer	Foraminifera	_	_	_	_	_	+		_
Arcella megastoma	Protozoa	+	+	+	+	+	-	+	-
Centropyxis aculeata	Protozoa	+	+	+	+	-	-	_	-
Bivalve	Bivalvia	_	-	_	_	_	-	_	+
Pyrgophorus coronatus	Gastropoda	_	-	+	+	+	+	+	+
<i>Bothriopupa</i> sp.	Gastropoda	_	-	_	_	+	+	+	+
Copepod	Copepoda	+	+	_	_	_	-	_	-
Darwinula stevensoni	Ostracoda	_	-	+	+	+	+	+	+
Cypridopsis vidua	Ostracoda	_	_	+	+	+	+	+	+
Pseudocandona sp.	Ostracoda	_	_	_	-	-	_	_	+
Cyprididae sp.	Ostracoda	_	_	_	-	-	_	_	+
Hygrobatidae sp.	Acari	+	+	_	-	-	_	_	_
Tyrphonothrus sp.	Acari	+	+	_	_	-	_	_	_
Chironomidae	Insecta	+	+	_	-	_	_	_	_



Figure 5. Non-metric multidimensional scaling plot based on Bray-Curtis index and squared root transformed abundance data of species from cenote X-Batún. Assemblage 1 (G1), assemblage 2 (G2), assemblage 3 (G3) and assemblage 4 (G4). Abbreviations are as follows: S – surface; OW – open water; CN – cavern; C – cave.

The SIMPER analysis was used to evaluate the contribution of each species to the Bray-Curtis associations. This analysis revealed that the taxa with higher contribution to the G1 assemblage, considering a cumulative percentage < 70%, were *Centropyxis aculeata*, *Arcella megastoma* y Chironomide. For G2 with a cumulative percentage of > 50%, Cypridopsis *vidua*, *Pyrgophorus coronatus* and *Bothriopupa* sp. were the most influential. For the G3 assemblage, *Darwinula stevensoni*, *Pyrgophorus coronatus* and *Cypridopsis vidua* were species with higher contribution with a cumulative percentage < 65%. On the G4 assemblage *Darwinula stevensoni*, and *Cypridopsis vidua* were the most influential (Table 3).

The ANOSIM test was then used to evaluate significant differences between the four habitat types identified in the NMDS. The ANOSIM test provides statistical support to determine that the four benthic assemblages are different in their composition and abundance (R = 0.99; p < 0.001). Ostracodes and gastropods for example, were mostly distributed toward the deepest zones of the cave, which is characterized by less light availability (Table 2). Chironomids, testate amoeba and copepods were distributed mainly toward the surface (Table 2).

Grupos	Especies	% de Contribución	% acumulado
G1	Centropyxis aculeata	35.75	35.75
	Arcella megastoma	20.64	56.39
	Chironomide	13.06	69.45
G2	Cypridopsis vidua	19.79	19.79
	Pyrgophorus coronatus	17.90	37.70
	Bothriopupa sp.	13.34	51.04
G3	Darwinula stevensoni	26.05	26.05
	Pyrgophorus coronatus	20.17	46.22
	Cypridopsis vidua	16.47	62.69
G4	Darwinula stevensoni	39.38	39.38
	Cypridopsis vidua	21.53	60.91

Table 3. SIMPER analysis results, showing the percentage of contribution of the species for the four ecological groups discriminated in the non-metric multidimensional scaling analysis.

Environmental variables

Vertical profiles of environmental variables at Cenote X-Batún show that conditions slightly change from surface to bottom, (Fig. 6). For temperature, the maximum variation is 0.5 °C, being the surface slightly warmer (27.9 °C) than bottom waters (27.4 °C). Similarly, pH displayed small changes along the water column, with overall neutral conditions and maximum variation of 0.05. TDS and conductivity show a similar behavior, with relatively low values at the first 10 meters and then progressively increasing to the bottom. For both variables, changes between surface and bottom conditions were relatively small. Dissolved oxygen shows a relatively small change from surface to bottom. Surface waters display about 5 mg/L, whereas bottom waters about 3.5 mg/L. In terms of dissolved oxygen availability, the entire system can be considered well oxygenated.



Figure 6. Water column environmental profiling of Cenote X-Batún: temperature, pH, total dissolved solids (TDS), conductivity and dissolved oxygen. Profiles are based on 350 measurements; black dots represent measured data.

Environmental influences on species assemblages of cenote X-Batún

Linear correlation demonstrated that light (r= -0.98, p= < 0.05) and total dissolved solids (r= -0.95, p= < 0.05) are more closely related to species assemblages determined in the NMDS.

The DCA, using environmental variables and species assemblages as supplementary variables, resulted in the following eigenvalues of the first two axes 0.76 and 0.08, respectively. Total inertia is 0.98. Cumulative percentage variance of speciesenvironment for the first two axes are 76.1 and 84.9%. Kendall Tau correlation coefficient (significant at 0.01 level) shows that first DCA axis was strongly related to pH (-0.99*), temperature (-0.97*), dissolved oxygen (-0.95*) and light (-0.84*) were the explicative variables of the data set. In the DCA ordination plot, species assemblage of the outer zone (G1) of the cenote were positioned together with explicative variables of the first axis. The G2 assemblage was positioned close to the center of the axis revealing that environmental variables have limited effect on the species composition. The G3 and G4, corresponded to deeper zones of the cenote, are positively influenced by TDS and negatively influenced by variables such as DO, temperature, pH and light (Fig. 7).



Figure 7. Detrended Correspondence Analysis (DCA) ordination diagram of benthic species and environmental variables in the cenote X-Batún. Statistical significance based on Monte Carlo test with forward selection $p \le 0.05$. Abbreviations are as follows: temperature (Temp), dissolved oxygen (DO), conductivity (Conductv), total dissolved solids (TDS). G1 assemblage of surface sediments; G2 assemblage of open water sediments; G3 assemblage of cavern; G4 assemblage of cave sediments identified in NMDS. Colors corresponded to that in the NMDS plot.

Discussion

Benthic species assemblages of Cenote X-Batún

Benthic biological composition of Cenote X-Batún was integrated by eight higher taxonomic groups and 15 species. In terms of species richness and diversity, the cenote was relatively homogenous, as ANOVA test did not find significant differences among zones. Evenness index, however, suggest that zones of the cenote were variable in terms of species abundance. The fauna of X-Batún based on Shannon diversity index is relatively low, however, considering species richness of subterranean environments globally, the fauna is relatively high. In Križna jama cave system (Slovenia, 8km long), for example 32 troglobitic aquatic taxa have been recorded so far (Polak and Pipan 2021).

In the American continent, San Marcos artesian well and the Edwards aquifer (United States, 10500 km²) display 55 aquatic taxa (Hutchins et al. 2021) and Toca do Gonçalo cave (0.5km long) and Areias cave systems (14km long) in Brazil display 22 and 28, troglobitic/stygobitic taxa respectively (Souza and Ferreira 2016).

Ostracodes were the group in which we identified the highest number of species with four. All ostracod species and genera of X-Batún are previously known as epigean, as they are mainly distributed in lakes of this region (Cohuo et al. 2017), but in the cenote ecosystem they all inhabit the aphotic zone. Presence of epigean ostracod species in cenotes either with direct sun light incidence or cavern type with partial light or complete darkness, have been recently observed in the Yucatán Peninsula (Macario-González et al. 2021). First explanations of this phenomenon postulates that geologically recent increase of the water table in the Yucatán Peninsula, driven by the last deglacial, produced new habitats in subterranean systems (Yokoyama et al. 2000; Mueller et al. 2010; Cohuo et al. 2018), which presumably were occupied by epigean taxa. The species *D. stevensoni* and the genus *Pseudocandona* are the unique ostracod groups that have previously been observed in subterranean environments in the Yucatán Peninsula (Cohuo et al. 2017; Macario-González et al. 2021) and elsewhere in the world (Sohn 1983; Namiotko 2004; Namiotko and Danielopool 2004).

Gastropods are another group restricted to the aphotic zone in X-Batún. *Pyrgophorus* is a genus recently observed in photic and aphotic zones of cenotes of Yucatán, likely associated to the chemosynthetic production in aphotic zones (Grego et al. 2019).

Other species of X-Batún such as mites, testate amoebae (van Hengstum et al. 2008), chironomids (Vinogradova and Riss 2007; Pérez et al. 2013) and copepods (Suárez-Morales and Reid 2003) are mainly associated to surface waters. These species are commonly observed in lakes of the Yucatán Peninsula and mainly associated to high organic matter content (Pérez et al. 2013).

Benthic assemblages change along cenote X-Batún

The NMDS analysis, based on the biological composition, distinguished four assemblages, that coincided with the surface, open water, cavern, and cave zones of the cenote. The deepest zones of the cenote, corresponding to complete darkness sites, display the higher species abundance. The ANOSIM test revealed significant differences between the four species assemblages, suggesting that in fact the four zones are biologically distinct. This implies that benthic species assemblages are confined to specific zones along the light gradient in X-Batún. We disregard invertebrate postmortem sediment transportation along the cenote, as taphonomy of transported shells are characterized by shell edge or ornamentation modifications, abrasion, bioerosion, internal and external incrustations, and high percentage of totally or partially broken shells (Lazo 2006; Chattopadhyay and Das 2013). All these characteristics lacking in our samples.

The deeper zones of the cenote, cavern and cave, which are characterized by lack or limited sun light availability was constituted by three taxonomic benthic groups and shown by the highest species abundance per gram of all sampling sites within the cenote. Ostracodes dominate the assemblage, the species *D. stevensoni* and *Cypridopsis* sp. show the higher statistical contribution to the assemblages as illustrated by the SIMPER analysis. Gastropods were also abundant in this zone almost equaling the abundance of *Cypridopsis* sp. Ostracodes are the animal group dominant in most sediment layers of epigean environments such as lakes and cenotes of the Yucatán Peninsula (Smirnov and Elías-Gutiérrez 2011; Cohuo et al. 2017; Macario-González et al. 2021). Previous surveys highlighted that aquatic systems in this region, without ostracodes are rare (Cohuo et al. 2017). Cenote X-Batún was previously considered a system without ostracodes because the practical absence of living specimens or valve remains in surficial sediments. The high abundance of ostracodes and gastropods in the aphotic zone, and its practical absence in surficial environments, is therefore a remarkable finding.

Ostracodes are capable to feed on a variety of forms and resources such as algae, bacteria and some of them are carnivores or detritivores (Martens and Horne 2009). In the aphotic zone of X-Batún characterized by very thin layer of sediment, covered mainly with calcite crystal plates, is challenged to determine food source for specimens. Given cenote morphology with a slope of about 150° from the entrance to the cave zone we speculate that food source is not dependent of allochthonous organic material only, but from other sources such a chemoautotrophy. At global scale, in cave environments have been observed that chemical compounds such as hydrogen sulfide (H2S) (Sarbu et al. 1996), methane and ammonium (Pohlman et al. 1997) can be synthetized by bacterial mats and transformed into organic products that become the main food source for subterranean organisms. In the Yucatán Peninsula, such processes have been observed in cenotes of coastal areas (which constitute subterranean estuaries), such as Ox Bel Ha Cave System (Brankovits et al. 2017). In cenote Bang belonging to this system, bacterial mats are capable trough methanotrophy, heterotrophy, and chemoautotrophy to make available organic compounds from methane and dissolved organic carbon which originated in the surface. The resulting products constitute the main food source for anchialine fauna (Brankovits et al. 2017).

In X-Batún, although bacterial structure, chemoautotrophy and subproducts have not already been quantified, the ecological pattern observed in benthic taxa i.e., the highest abundance in aphotic zone, suggests that there may exist interactions between bacterial activity and the benthic community. Similar patterns of benthic species distributions were observed in other freshwater cenotes of the Ring of Cenotes, such as Xoch, Tzitzila and Dzontila. There, the presence of gastropods in the light limited or aphotic zones was assumed as the result of interaction with chemolithotrophic bacterias or organic products resulting from chemolithotrophic processes (Grego et al. 2019). These biological patterns observed at regional scale in the Peninsula therefore, highlights that the bacterial and subterranean species relationships observed in subterranean estuaries such as in Ox Bel Ha, may be widespread in Yucatán and occurring as well in freshwater cenotes.

We hypothesized that the unique presence of epigean ostracodes in the aphotic zone of X-Batún represent an ecological adaptation, resulting from active movements of the species from highly competitive zones in the surface to food rich and low competitive environment in the aphotic zone. The SIMPER test revealed that the groups with higher statistical contribution to outer cenote zones, surface and open waters, were testate amoebae, copepods and mites. These taxa commonly inhabit lake environments and particularly zones where sun light is fully available, and primary productivity is high (Sigala et al. 2016; Charqueño et al. 2019; Montes-Ortiz and Elías-Gutiérrez 2020). This illustrates that surface waters of X-Batún have a biological structure typical of the epigean environments. Comparatively, surficial fauna of X-Batún is similar to that from other oligotrophic cenotes in Yucatán Peninsula, except for the absence of ostracodes in surficial sediments (Smirnov and Elías-Gutiérrez 2011).

Influence of environmental variables of Cenote X-Batún over benthic assemblages

The measured environmental variables of cenote X-Batún show that there exists little variation from the upper layers to bottom. Light was the variable with more drastic changes as it varied from full incidence in surface and open waters to twilight in the cavern zone and complete darkness in the cave. Relatively homogeneous conditions in this system, can be expected, as it is primarily subterranean. Subterranean environments tend to be physically and chemically stable (Hüppop 2012). Particularly, in X-Batún, only a relatively little area (<0.1 hc) is interacting with the epigean environments, and it is protected from wind and sun light by a dense canopy. Thus, drastic changes of water column physical or chemical conditions are not expected, even at surface waters. Linear correlation however, determined that light and TDS were the most influential for species assemblages. At regional scale, sun light is known as primary factor structuring biological compositions, because the full availability at surface is monotonically decreasing toward the deepest parts of the cave, changing the productivity and food webs (Culver and Pipan 2009; Hüppop 2012; Borisov et al. 2021). The DCA analysis, however, remarks that environmental variables exert different influences on the biological assemblages along the cave. The single entrance of the cenote, for example, characterized by full sun light incidence and high detritus input, support a typical epigean benthic species assemblages associated to higher values of pH, temperature and, dissolved oxygen (Vinogradova and Riss 2007; van Hengstum et al. 2008; Pérez et al. 2013). Open water assemblage, featuring species from surface and deeper waters, seems to be little influenced by environmental variables, as it was located close to the center of the axes 1 and 2. This is expected as the variables are more stable in this zone, light is fully available and therefore autochthonous productivity seem to be the main source of food. The G3 and G4, corresponded to deeper zone of the cenote show a negative correlation with most of the environmental variables, as the values of most of them decrease. Positive correlations were recovered from conductivity and TDS. These species assemblages are similar in structure and mainly associated to zones where sun light is limited or lacking. In the NMDS, given the patterns of species assemblages and their influence by environmental variables, we deduce that sun light is the main driver for species distribution in X-Batún, which is also supported by linear correlation.

Conclusions

There is only scattered information available about the diversity, distribution patterns, colonization methods and ecology of the benthic assemblages in subterranean realms of the Yucatán Peninsula. We characterized the benthic fauna of a Yucatán water-filled doline and its submerged cave. The fauna was composed of eight higher taxonomic groups and four assemblages. The deepest, fully dark zones of the cave displayed the highest species diversity and abundance, which raises the possibility of chemoautotrophy. Environmental variables of the different zones of the water column display a little variation from the upper layers to bottom. Linear regression demonstrated that light gradient is the most relevant for species assemblages. Temperature, pH, dissolved oxygen, and light were the most meaningful for individual assemblage as evidenced by the DCA. This study highlights the importance of environmental gradients in structuring benthic biological communities in subterranean systems and the existence of complex biological interactions mediated by different sources of energy in the Yucatán Peninsula.

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RESEARCH ARTICLE



Two new species add to the diversity of *Eoniphargus* in subterranean waters of Japan, with molecular phylogeny of the family Mesogammaridae (Crustacea, Amphipoda)

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Abstract

Amphipod crustaceans are a major group of invertebrates that predominantly occur in groundwater ecosystems. *Eoniphargus* is a mesogammarid genus with only two known species from the groundwater systems of the Japanese archipelago and Korean Peninsula. However, there is a dearth of taxonomic studies on this genus, and the species diversity within *Eoniphargus* is unclear. Here, we describe two new species, *E. iwataorum* **sp. nov.** and *E. toriii* **sp. nov.**, collected from the interstitial waters in Tochigi and Shizuoka Prefectures in the Japanese archipelago. These two new species are distinguished from their congeners by the following features: head, urosomite 3, first and second antennae, mandibles, and maxilla 1. *Eoniphargus kojimai* is redescribed here based on material collected near the type locality. Molecular phylogenetic analyses based on the nuclear 28S rRNA and mitochondrial COI genes revealed that *E. kojimai* is sister to *E. iwataorum* **sp. nov.** In this study, we also briefly discuss the phylogenetic relationships of Mesogammaridae based on the molecular phylogenetic analyses.

Keywords

Cryptic species, interstitial water, molecular phylogeny, new species, taxonomy

Introduction

Compared to epigean ecosystems, subterranean environments are generally stable (Badino 2010). Owing to the absence of light, subterranean habitats are almost entirely dependent on external energy, which is mostly scarce (Poulson and Lavoie 2000). Therefore, subsurface habitats usually have low biomass and species diversity (Holsinger 1988; Cardoso 2012). Several species show limited distribution as a result of isolation and adaptation to underground environments (Culver et al. 2000). Crustaceans, segmented worms, flatworms, rotifers, water mites, and larvae of aquatic insects are predominant in subterranean waters, but insects are rarely found in cave waters (Boulton et al. 1998; Sket 1999). Amphipod crustaceans constitute a major portion of the aquatic organisms of groundwater ecosystems and have been reported from a variety of subterranean environments, including caves, interstitial riverbed waters, and springs (Holsinger 1993).

Eoniphargus Uéno, 1955 was established by Uéno (1955) as a new subgenus of Neoniphargus Stebbing, 1899. This subgenus was subsequently elevated to the genus level by Straškraba (1964). The affiliation of this genus to a family is controversial. Straškraba (1964) included Eoniphargus in Gammaridae Leach, 1814. Bousfield (1977) moved this genus to the newly established Neoniphargidae family. However, neither Barnard and Barnard (1983) nor Holsinger (1994) assigned Eoniphargus to a specific family, owing to the unclear nature of its diagnostic traits. A taxonomic revision of Eoniphargus by Tomikawa et al. (2007), based on molecular phylogenetic analyses, included this genus in Mesogammaridae Bousfield, 1977. Mesogammaridae currently include two marine genera, Mesogammarus Tzvetkova, 1965 and Paramesogammarus Bousfield, 1979, and four subterranean genera that occur in freshwaters, Eoniphargus, Indoniphargus Straškraba, 1967, Octopupilla Tomikawa, 2007, Potiberaba Fišer, Zagmajster & Ferreira, 2013; however, the monophyly of the family remains to be tested using molecular methods (see Sidorov et al. 2018 for alternative opinion on Indoniphargus). Eoniphargus currently comprises two species: E. kojimai Uéno, 1955 from the subterranean waters in Tokyo and Shizuoka and E. glandulatus Stock & Jo, 1990 from cave pools in the Korean Peninsula (Uéno 1955; Stock and Jo 1990; Tomikawa et al. 2007). In recent years, molecular phylogenetic analyses using DNA sequences have greatly advanced our understanding of the phylogenetic relationships of amphipods (Hou et al. 2011, 2014; Copilaș-Ciocianu et al. 2020). However, the phylogenetic position of Mesogammaridae and phylogenetic relationships within the family are not fully understood.

The authors recently obtained several specimens of *Eoniphargus* from interstitial waters in Japan. Detailed morphological observations and molecular phylogenetic analyses of these samples have revealed the presence of two previously undescribed species. Here, we describe these two novel species and redescribe *E. kojimai* obtained near the type locality.

Methods

Sampling and morphological observation

Specimens of *Eoniphargus* were collected from four localities in Japan (Fig. 1). The specimens were collected by washing out gravel from the bottom of rivers and springs using fine-mesh hand nets and fixed in 99% ethanol on-site.

All appendages were dissected by fine needles in 80% ethanol under a stereomicroscope (Olympus SZX7) and mounted in gum-chloral medium on glass slides. Slides were examined using a light microscope (Nikon Eclipse Ni), with appendages illustrated using a camera lucida.

Type specimens are deposited at the National Museum of Nature and Science, Tsukuba (**NSMT**).



Figure 1. Map showing the collection localities of the specimens examined in this study. Names of localities are shown in Table 1.

Species	Voucher or isolate	Locality	NCBI GenBank acc. nos.		
_	#		28S rRNA	COI	
Acanthogammaridae					
Dorogostaiskia parasitica	MZH:112037	Lake Baikal, Russia	KF586548	KF586540	
Anisogammaridae					
Jesogammarus hebeiensis	294	Beijing, China	EF582998	KT180186	
Gammaracanthidae					
Gammaracanthus lacustris	SLOCHN141	Savonranta Munic, Finland	JF965829	JF965997	
Gammaracanthu loricatus	SLOCHN171	Spitsbergen, Norway	JF965830	JF965998	
Gammarellus angulosus	RBINS-INV.132647	Westkapelle, Netherland	KT808715	FJ581638	
Gammaridae					
Anopogammarus revazi	SLOCHN245	Martvili, Georgia	KF478431	KF478522	
Barnardiorum shadini	SLOCHN263	Hodža Obi-Garm, Tajikistan	JF965826	JF965994	
Chaetogammarus ischnus	SLOCHN051	Babadag, Romania	KF478441	KF478532	
Dikerogammarus villosus	SLOCHN052	Babadag, Romania	KF478442	KF478533	
Echinogammarus acarinatus	SLOCHN082	Mostar, Bosnia and Herzegovina	KF478458	KF478548	
Gammarus lacustris			EF582964	EF570317	
G. mukudai	G857	Katsumoto, Iki, Nagasaki, Japan	AB893233	AB893343	
G. nipponensis	G621	Ukyo, Kyoto, Japan	AB893226	AB893336	
G. tigrinus	609	Netherland	EF582994	EF570348	
Jugogammarus kusceri	SLOCHN073	Krka, Slovenia	KF478462	KF478552	
Rhipidogammarus rhipidiophorus	SLOCHN162	Dorgali, Sardegna, Italy	JF965823	JF965991	
Mesogammaridae				-	
Eoniphargus iwataorum sp. nov.	NSMT-Cr 30783	Sabi River, Imaizumi, Ohtawara,	LC709238*	LC709248*	
1 0 1		Tochigi Prefecture, Japan (1)			
E. kojimai	G1750	Mamashita Spring, Kunitachi,	LC709239*	LC709249*	
E. kojimai	NSMT-Cr 30787	Hino Tokyo, Japan (2) Hino Tokyo Japan (3)	LC709240*	LC709250*	
E. kojimai	NSMT-Cr 30788	Hino, Tokyo, Japan (3) Hino, Tokyo, Japan (3)	LC709241*	LC709251*	
<i>E. toriii</i> sp. nov.	G56	Seto River, Terajima, Fujieda, Shizuoka, Japan (4)	LC709242*	LC709252*	
E. toriii sp. nov.	G71	Seto River, Terajima, Fujieda, Shizuoka, Japan (4)	LC709243*	LC709253*	
Mesogammarus melitoides	G86	Muroran, Hokkaido, Japan (5)	LC719002*	NA	
Octopupilla felix	G54	Koza River, Wakayama, Japan (6)	LC719003*	LC719248*	
Pontogammaridae	-				
Obesogammarus crassus	SLOCHN055	Babadag, Romania	KF478445	KF478536	
Paraniphargoides motasi	SLOCHN188	Gilan, Iran	KF478485	KF478571	
Pontogammarus robustoides	SLOCHN255	Delta Volgi, Russia	IF965822	IF965990	
Stenogammarus similis	SLOCHN187	Gilan, Iran	KF478484	KF478570	
Turcogammarus spandli	SLOCHN026	Thessaloniki, Greece	KF478437	KF478528	
Typhlogammaridae					
Metohia carinata	SLOCHN019	Rijeka Crnojevica. Montenegro	KF478498	KF478584	
Typhlogammarus mrazeki	SLOCHN113	Cetinie, Montenegro	KF478504	KF478590	
Zenkevitchia admirahilis	SLOCHN200	Gudauta, Georgia	KF478514	KF478599	
Outgroup	0200111200	Guandan, Georgia			
Pseudocrangonyx vezonis	KUZ Z1969	Daisen, Akita, Japan	LC17151	LC171519	
0 7 7		,, ,			

Table 1. Samples used for molecular analyses with voucher/isolate number, collection locality, and NCBI
 GenBank accession number. Sequences marked with an asterisk (*) were obtained for the first time in this study.

Molecular phylogenetic analyses

Genomic DNA was extracted from the appendage muscle of the specimens following procedures detailed by Tomikawa et al. (2014a, b). The primer sets for PCR and cycle sequencing reactions used in this study were as follows: for 28S rRNA (28S), 28SF and 28SR (Tomikawa et al. 2012); and for cytochrome c oxidase subunit I (COI), LCO1490 and HCO2198 (Folmer et al. 1994). PCR and DNA sequencing were performed using the method detailed by Tomikawa (2015). In total, 15 sequences were newly obtained and deposited in the International Nucleotide Sequence Databases (**INSD**) through the DNA Data Bank of Japan (**DDBJ**) (Table 1).

In addition to the newly obtained sequences, 50 sequences of 24 gammaroid species and one crangonyctoid *Pseudocrangonyx yezonis* Akatsuka & Komai, 1922, which was selected as the outgroup, were obtained from the INSD in accordance with the previous studies (Hou et al. 2011, 2014; Copilaş-Ciocianu et al. 2020), and were included in the present phylogenetic analyses (Table 1). The phylogenetic analyses were conducted based on sequences of nuclear 28S and mitochondrial COI. The alignment of COI was trivial, as no indels were observed. The 28S sequences were aligned using the Muscle algorithm implemented in MEGA X (Kumar et al. 2018). The lengths of the 28S and COI were 892 and 658 bp.

Phylogenetic relationships were reconstructed via Maximum Likelihood (ML) and Bayesian Inference (BI). The best evolutionary models were selected based on the corrected Akaike Information Criterion (AIC) for ML using MEGA X (Kumar et al. 2018). ML phylogenies were conducted using MEGA X (Kumar et al. 2018) under the substitution model GTR+G, and 1000 bootstrap replications (Felsenstein 1985) were performed to estimate statistical support for branching patterns. BI and Bayesian posterior probabilities (PPs) were estimated using MrBayes v. 3.2.5 (Ronquist et al. 2012). The best-fit partition scheme and models for each partition were selected with the Bayesian information criterion using PartitionFinder with the "greedy" algorithm: for 28S, SYM+G; for COI 1st position, HKY+G; for COI 2nd position, HKY+I+G; for COI 3rd positions, GTR+I+G. Two independent runs of four Markov chains were conducted for 10 million generations, and the tree was sampled every 100 generations. The parameter estimates and convergence were checked using Tracer v. 1.7.1 (Rambaut et al. 2018), and the first 50001 trees were discarded based on the results.

Results

Family Mesogammaridae Bousfield, 1977

Genus Eoniphargus Uéno, 1955

Japanese name: Chikayokoebi-zoku

Neoniphargus (Eoniphargus) Uéno, 1955: 148.

Eoniphargus: Straškraba, 1964, 136, 138; Straškraba 1967, 127; Bousfield 1977, 301; Barnard and Barnard 1983, 581–582; Holsinger 1994, 157; Tomikawa et al. 2007, 646.

Diagnosis. Head without eyes, rostrum short, inferior antennal sinus distinct. Pleonites 1–3 with dorsal setae, lacking processes. Urosomite 2 with dorsal robust setae. Antenna 1 longer than antenna 2; accessory flagellum three- or four-articulate. Male antenna 2 with calceoli. Mandible with triturative molar with seta; left incisors five- or six-dentate; left lacinia mobilis four- or five-dentate; palp article 3 with A-, D- and E-setae. Maxilla 1 with inner plate bearing plumose setae; outer plate with 11 serrate robust setae. Inner plate of maxilla 2 with oblique row of facial setae. Gnathopods subchelate. Coxa of pereopod 4 with posterior concavity. Coxal gills with stalks, on gnathopod 2 and pereopods 3–6. Uropod 1 with basofacial robust setae on peduncle. Uropod 3 with small, scale-like inner ramus; outer ramus one- or two-articulate. Telson cleft with apical robust setae.

Type species. Neoniphargus (Eoniphargus) kojimai Uéno, 1955, original designation.

Remarks. Eoniphargus is morphologically similar to Octopupilla in the presence of pleonites without dorsal processes, five-dentate incisor of the left mandible, mandibular palp article 3 with A-setae, inner plate of maxilla 2 with oblique row of facial setae, stalked coxal gills, peduncle of uropod 1 with basofacial robust setae, and telson lobes tapering distally. However, Eoniphargus differs from Octopupilla in the following features (features of Octopupilla in parentheses): eyes absent (rudimentary eyes), incisor of right mandible five- to six-dentate (four-dentate), inner lobes of the lower lip absent (vestigial), and inner ramus of uropod 3 shorter than $0.3 \times$ the outer ramus (0.7 ×). *Eo*niphargus also resembles Indoniphargus Straškraba, 1967, but the familial affiliation of the latter has been controversial. Tomikawa et al. (2007) and Fišer et al. (2013) included Indoniphargus in Mesogammaridae. Sidorov et al. (2018) regarded Indoniphargus as a member of Austroniphargidae Iannilli, Krapp, & Ruffo, 2011. However, since the taxonomic position of *Indoniphargus* is beyond the scope of this paper, it will not be discussed here further. Eoniphargus is distinguished from Indoniphargus by the presence of facial setae in an oblique row of the inner plate of maxilla 2 (absent in *Indoniphargus*) and the elongate propodi of gnathopods 1 and 2 (mitten-form in *Indoniphargus*).

Eoniphargus toriii sp. nov.

https://zoobank.org/2A3DFB47-9FEC-46E4-A003-923CCD93E20D Figs 2–5 New Japanese name: Torii-chikayokoebi

Eoniphargus kojimai: Tomikawa et al. 2007, 647, figs 2-6.

Type material. *Holotype*: 3 4.3 mm (NSMT-Cr 16652), Seto River (34.880555°N, 138.218888°E), Terajima, Fujieda, Shizuoka Prefecture, Japan, collected by T. Torii on 3 June 2004. *Paratypes*: 2 9, 4.6 mm and 4.3 mm (NSMT-Cr 16653 and 16654), data same as for the holotype.

Diagnosis. Urosomite 3 without dorsal setae. Epimeral plates 2–3 without ventral setae. Peduncular article 1 of antenna 1 with robust seta on posterodistal corner. Antenna 2 with peduncular article 2 gland cone not exceeding end of article 3; calceoli present in male. Mandible with 5-dentate left incisor; left lacinia mobilis 4-dentate, right one bifid with three or four teeth. Inner plate of maxilla 1 with six plumose setae. Inner plate of maxilla 2 with seven facial seta in oblique row. Peduncle of pleopod 3 with seta. Uropod 1 with peduncle bearing basofacial robust setae. Uropod 3 with inner ramus 0.3 times as long as outer ramus; outer ramus two-articulate, with plumose setae on medial margin. Telson length 1.1 times width, cleft for 67% of length.

Etymology. The new species is named after Dr. Takaaki Torii, who collected the species.

Description. Male holotype, NSMT-Cr 16652. Head (Fig. 2A) as long as pereonites 1 and 2 combined; eyes absent; inferior antennal sinus distinct with rounded angle. Dorsal margins of pleonites 1–3 (Figs 2A, 5A–C) each with four setae. Epimeral plates 1–3 (Figs 2A, 5N–P) with weakly pointed posterodistal corners; ventral margins without setae; posterior margins of plates 1–3 with one, zero, one seta, respectively. Dorsal margins of urosomites 1 and 2 with robust seta, urosomite 3 dorsally bare.

Antenna 1 (Figs 2A, 3A) length 0.6 times as long as body; length ratio of peduncular articles 1–3 in 1.0 : 0.8 : 0.4; posterodistal corner of peduncular article 1 with robust seta; accessory flagellum three-articulate (Fig. 3B); primary flagellum with approximately 22 articles, each article with one aesthetasc (Fig. 3C). Antenna 2 (Figs 2A, 3D) length 0.6 times as long as antenna 1; peduncular article 4 length 0.9 times as long as article 5; right peduncular article 5 with calceoli, left one lacking calceoli; flagellum 16-articulate, with calceoli (Fig. 3E).

Upper lip (Fig. 2B) with rounded apical margin bearing fine setae. Mandibles (Fig. 2C–E) with left and right incisors comprising five and five–six teeth, respectively; left lacinia mobilis comprising four teeth, right lacinia mobilis bifd with three teeth; molar process triturative with plumose seta; length ratio of palp articles 1–3 in 1.0 : 2.4 : 1.9; palp article 1 without setae; palp article 2 with eight marginal setae; palp article 3 with pair of A-setae, many D-setae and E-setae, lateral face with fine setae. Lower lip (Fig. 2G) lacking inner lobes; outer lobes broad, shoulder rounded, with fine setae. Maxilla 1 (Fig. 2H–J) with triangular inner lobe, bearing six plumose setae on medial margin; outer plate rectangular, with 11 serrate robust setae apically; palp 2-articulate, article 1 without setae, article 2 with six robust setae apically. Maxilla 2 (Fig. 2K) with inner plate bearing seven plumose setae in oblique row. Maxilliped (Fig. 2L–O) with inner plate reaching end of palp article 1, subquadrate, bearing three subapical robust setae and medial robust seta; outer plate ovate, reaching middle of palp article 2, with row of robust setae along apical to medial margins; palp 4-articulate, article 2 longest with medial setae, nail of article 4 distinct.

Gnathopod 1 (Fig. 3J, K) with subquadrate coxa bearing setae along anterior to ventral margins; anterior and posterior margins with long setae; carpus length 0.9 times as long as length of propodus and 1.5 times width of carpus; propodus length 1.9 times width, palmar margin weakly serrate, oblique, with three medial and two lateral



Figure 2. *Eoniphargus toriii* sp. nov., male holotype (NSMT-Cr 16652), female paratype (NSMT-Cr 16653) **A** habitus, lateral view **B** upper lip, anterior view **C** left mandible, medial view **D** incisor and lacinia mobilis of left mandible, medial view **E** and **F** incisor and lacinia mobilis of right mandible, medial view **G** lower lip, anterior view **H** maxilla 1, dorsal view **I** outer plate of maxilla 1, dorsal view **J** palp of maxilla 1, dorsal view **K** maxilla 2, dorsal view **L** maxilliped, dorsal view **M** palp article 4 of maxilliped, dorsal view **N** outer plate of maxilliped, dorsal view **F**, **P** female; others, male. Modified from Tomikawa et al. (2007).



Figure 3. Eoniphargus toriii sp. nov., male holotype (NSMT-Cr 16652), female paratype (NSMT-Cr 16653) A antenna 1, medial view B accessory flagellum of antenna 1, medial view C aesthetasc on flagellar article of antenna 1, medial view D antenna 2 (some flagellar articles are omitted), medial view E calceolus on flagellar article of antenna 2, medial view F antenna 1 (female, some flagellar articles are omitted), medial view G accessory flagellum of antenna 1 (female), medial view G accessory flagellum of antenna 1 (female), medial view H aesthetasc on flagellar article of antenna 1 (female), medial view I antenna 2 (female, some flagellar articles are omitted), medial view J gnathopod 1, lateral view K palmar margin and dactylus of gnathopod 1 (some setae are omitted), lateral view L gnathopod 2 (lemale), lateral view O palmar margin and dactylus of gnathopod 1 (female), lateral view P gnathopod 2 (female), lateral view Q palmar margin and dactylus of gnathopod 2 (female, some setae are omitted), lateral view F-H, N-Q female; others, male. Modified from Tomikawa et al. (2007).



Figure 4. *Eoniphargus toriii* sp. nov., male holotype (NSMT-Cr 16652), female paratype (NSMT-Cr 16653) **A** pereopod 3, lateral view **B** dactylus of pereopod 3, lateral view **C** coxa of pereopod 3 (female), lateral view **D** pereopod 4, lateral view **E** dactylus of pereopod 4, lateral view **F** coxa of pereopod 4 (female), lateral view **G** pereopod 5, lateral view **H** dactylus of pereopod 5, lateral view **I** coxa–merus of pereopod 5 (female), lateral view **J** pereopod 6, lateral view **K** dactylus of pereopod 6, lateral view **L** coxa–merus of pereopod 6 (female), lateral view **M** coxa–merus of pereopod 7, lateral view **N** coxa–merus of pereopod 7 (female), lateral view **C**, **F**, **I**, **L**, **N** female; others, male. Modified from Tomikawa et al. (2007).



Figure 5. *Eoniphargus toriii* sp. nov., male holotype (NSMT-Cr 16652), female paratype (NSMT-Cr 16653) A–C dorsal margins of pleonites 1–3, dorsal views D–F dorsal margins of urosomites 1–3, dorsal views G pleopod 1 (plumose setae on rami are omitted), medial view H retinacula of pleopod 1, medial view I bifid setae of pleopod 1, medial view J pleopod 2 (plumose setae on rami are omitted), medial view K pleopod 3 (plumose setae on rami are omitted), medial view L pleopod 1 (female, rami are omitted), medial view M pleopod 2 (female, rami are omitted), lateral view N–P epimeral plates 1–3, lateral views Q epimeral plate 2 (female), lateral view R uropod 1, dorsal view S uropod 2, dorsal view T uropod 2 (female), of the setae of uropod 3, ventral view V terminal article of outer ramus of uropod 3, ventral view W uropod 3 (female), ventral view X telson, dorsal view Y telson (female), dorsal view L, M, Q,T, W,Y female; others, male. Modified from Tomikawa et al. (2007).

robust setae; dactylus not reaching posterodistal corner of propodus. Gnathopod 2 (Fig. 3L, M) with coxa expanded proximally bearing setae along anterior to ventral margins; posterior margin of basis with long setae; carpus length 1.2 times as long as length of propodus and 2.8 times width of carpus; propodus length 2.4 times width, palmar margin weakly serrate, almost vertical, with two medial and two lateral robust setae; dactylus not reaching posterodistal corner of propodus.

Pereopod 3 (Fig. 4A, B) with subquadrate coxa, proximally expanded, bearing setae along anterior to ventral margins; posterior margin of basis with long setae. Pereopod 4 (Fig. 4D, E) with coxa bearing setae along anterior to ventral margins, posteroproximally concave; posterior margin of basis with long setae. Pereopod 5 (Fig. 4G, H) with bilobed coxa bearing seta on anterior and posterior lobes; anterior margin of basis with robust setae, posterodistal corner weakly lobate. Pereopod 6 (Fig. 4J, K) with bilobed coxa bearing seta on posterior lobe; basis ovate, with robust setae on anterior margin, posterodistal corner weakly lobate. Pereopod 7 (Fig. 4M) with subtriangular coxa bearing seta on posterior margin; basis elliptical, with robust setae on anterior margin, posterodistal corner weakly lobate.

Coxal gills (Figs 3L, 4A, D, G, J) ovate with stalks on gnathopod 2 and pereopods 3–6.

Pleopods 1–3 (Fig. 5G–K) with peduncles longer than rami, bearing distal seta on pleopod 2 and proximal seta on pleopod 3; retinacula paired (Fig. 5H); mediobasal margin of inner ramus with bifid plumose setae (Fig. 5I); rami well developed.

Uropod 1 (Fig. 5R) with peduncle bearing three basofacial robust setae and dorsal robust setae; inner ramus length 0.7 times as long as peduncle, with two medial and one lateral robust setae, and ventroproximal robust seta; outer ramus length 0.9 times as long as inner ramus, with two robust setae on medial margin. Uropod 2 (Fig. 5S) with peduncle bearing dorsal robust setae; inner ramus length 0.8 times as long as peduncle, bearing two medial robust setae and two ventroproximal slender setae; outer ramus length 0.6 times as long as inner ramus, without marginal setae. Uropod 3 (Fig. 5U, V) with peduncle length 0.3 times as long as outer ramus, with two robust setae on distal edge; inner ramus length 0.3 times as long as outer ramus, with two robust setae on medial margin; outer ramus 2-articulate, proximal article with plumose setae on medial margin and robust setae on medial and lateral margins, terminal article length 0.3 times as long as proximal article with subapical setae.

Telson (Fig. 5X) length 1.1 times width, each lobe with two lateral and three apical robust setae and two dorsolateral penicillate setae, cleft for 67%.

Female paratype, NSMT-Cr 16653. Antenna 1 (Fig. 3F–H) with peduncles 1–3 of which length ratio in 1.0 : 0.8 : 0.5; accessory flagellum 4-articulate (Fig. 3G). Antenna 2 (Fig. 3I) without calceoli.

Incisor of right mandible 6-dentate (Fig. 2F); right lacinia mobilis 4-dentate, weakly bifid. Apical robust setae of inner plate of maxilliped stiffer than those of male (Fig. 2P).

Gnathopod 1 (Fig. 3N, O) with carpus length 1.8 times width; length of propodus 2.0 times width; dactylus reaching posterodistal corner of propodus. Gnathopod 2 (Fig. 3P, Q) with dactylus reaching posterodistal corner of propodus.

Brood plates (Figs 3P, 4I) narrow, lacking setae, on gnathopod 2 and pereopods 3–5. Peduncles of pleopods 1 and 3 with three and one setae, respectively (Fig. 5L, M).

Uropod 2 (Fig. 5T) with inner ramus bearing two medial and one lateral robust setae; outer ramus with medial robust seta. Uropod 3 (Fig. 5W) with terminal article of outer ramus length 0.2 times as long as proximal article.

Telson (Fig. 5Y) as long as wide, cleft for 71%.

Distribution and environment. The species is known only from its type locality in the Seto River, Shizuoka Prefecture, Japan. Specimens were collected at a depth of 20 cm, from the bank of the river.

Remarks. *Eoniphargus toriii* sp. nov. is similar to *E. kojimai* and *E. iwataorum* sp. nov., with a head bearing deep antennal sinus, antenna 1 peduncular article 1 with a robust seta on the posterodistal corner, antenna 2 peduncular article 2 with a gland cone not exceeding peduncular article 3, uropod 1 peduncle with basofacial robust setae, and uropod 3 with 2-articulate outer ramus. However, *E. toriii* sp. nov. differs from *E. kojimai* and *E. iwataorum* sp. nov. in the following features: right mandible with lacinia mobilis bearing three or four teeth (more than four teeth in *E. kojimai* and *E. iwataorum* sp. nov.) and maxilla 1 having six medial setae on the inner plate (eight medial setae in *E. kojimai* and *E. iwataorum* sp. nov.).

Eoniphargus iwataorum sp. nov.

https://zoobank.org/4234A07E-026A-4DB1-A2E8-2DE845E6295E Figs 6–8 New Japanese name: Iwata-chikayokoebi

Type material. *Holotype*: \bigcirc 5.6 mm (NSMT-Cr 30782), Sabi River (36.898181°N, 140.012153°E), Imaizumi, Ohtawara, Tochigi Prefecture, Japan, collected by Y. Iwata on 27 January 2019. *Paratype*: \bigcirc 5.3 mm (NSMT-Cr 30783; G1751), \bigcirc 6.0 mm (NSMT-Cr 30784), data same as for the holotype.

Diagnosis. Urosomite 3 without dorsal setae. Epimeral plates 2–3 each with ventral robust seta. Peduncular article 1 of antenna 1 with robust seta on posterodistal corner. Antenna 2 with peduncular article 2 gland cone not exceeding end of article 3. Mandible with 5-dentate left incisor; left lacinia mobilis 4-dentate, right one bifid with many teeth. Inner plate of maxilla 1 with eight plumose setae. Inner plate of maxilla 2 with seven facial seta in oblique row. Peduncle of pleopod 3 with seta. Uropod 1 with peduncle bearing basofacial robust setae. Uropod 3 with inner ramus 0.25 times as long as outer ramus; outer ramus 2-articulate, with plumose setae on medial margin. Telson almost as long as width, cleft for 67% of length.

Etymology. The specific epithet was derived from the names of Mr. Yasuyuki Iwata and Mr. Tomofumi Iwata, who collected the specimens of this new species.

Description. Female holotype, NSMT-Cr 30782. Head (Fig. 6A) as long as pereonites 1 and 2 combined; eyes absent; inferior antennal sinus distinct with rounded angle. Dorsal margins of pleonites 1–3 (Fig. 6B–D) each with four setae. Posterodistal corners of epimeral plates 1–3 (Fig. 6E–G) weakly produced; ventral margin of epimeral plate 1 without setae, epimeral plates 2 and 3 each with ventral robust seta; posterior margins of epimeral plates 1–3 each with two setae. Dorsal margin of uro-somite 1 (Fig. 6H) with slender setae; urosomite 2 (Fig. 6I) with dorsal robust setae; urosomite 3 (Fig. 6J) without dorsal setae.

Antenna 1 (Fig. 6K) length 0.25 times as long as body; length ratio of peduncular articles 1–3 in 1.0 : 0.8 : 0.4; posterodistal corner of peduncular article 1 with robust seta; accessory flagellum 4-articulate; primary flagellum 27-articulate. Antenna 2 (Fig. 6L) length 0.7 times as long as antenna 1; peduncular article 4 slightly shorter than article 5; flagellum 22-articulate; calceoli absent.

Upper lip (Fig. 6M) with rounded apical margin bearing fine setae. Mandibles (Fig. 6N–P) with left and right incisors comprising five and six teeth, respectively; left lacinia mobilis comprising four teeth, right lacinia mobilis bifd with many teeth; molar process triturative with plumose seta; length ratio of palp articles 1–3 in 1.0 : 2.0 : 1.5; palp article 1 without setae; palp article 2 with eight marginal setae; palp article 3 with pair of A-setae, several D-setae and E-setae, lateral face with fine setae. Lower lip (Fig. 6Q) lacking inner lobes; outer lobes broad, shoulder rounded, with fine setae. Maxilla 1 (Fig. 6R, S) with elliptical inner lobe, bearing eight plumose setae on medial margin; outer plate rectangular, with 11 serrate robust setae apically; palp 2-articulate, article 1 without setae, article 2 with five robust setae and slender plumose seta apically. Maxilla 2 (Fig. 6T) with inner plate bearing seven plumose setae in oblique row. Maxilliped (Fig. 7A–C) with inner plate exceeding end of palp article 1, subquadrate, bearing three subapical robust setae and medial robust seta; outer plate ovate, reaching middle of palp article 2, with row of robust setae along apical to medial margins; palp 4-articulate, article 2 longest with medial setae, nail of article 4 distinct.

Gnathopod 1 (Fig. 7D, E) with subquadrate coxa bearing setae along anterior to ventral margins; anterior and posterior margins of basis with long setae; carpus length 0.9 times as long as length of propodus and 1.4 times width of carpus; propodus length 1.7 times width, palmar margin weakly serrate, oblique, with three medial and one lateral robust setae; dactylus reaching posterodistal corner of propodus. Gnathopod 2 (Fig. 7F, G) with coxa expanded proximally bearing setae along anterior to ventral margins; posterior margin of basis with long setae; carpus length 1.2 times as long as length of propodus and 2.8 times width of carpus; propodus length 2.2 times width, palmar margin weakly serrate, almost vertical, with two medial and two lateral robust setae; dactylus reaching posterodistal corner of propodus.

Pereopod 3 (Fig. 7H, I) with subquadrate coxa, proximally expanded, bearing setae along anterior to ventral margins; posterior margin of basis with long setae. Pereopod 4 (Fig. 7J) with coxa bearing setae along anterior to ventral margins, posteroproximally concave; posterior margin of basis with long setae. Pereopod 5 (Fig. 7K) with bilobed coxa bearing seta on posterior lobe; anterior margin of basis with robust setae, postero-distal corner weakly lobate. Pereopod 6 (Fig. 7L) with bilobed coxa bearing seta on anterior lobes; basis with robust setae on anterior margin, posterodistal corner weakly lobate. Pereopod 7 (Fig. 8A–C) with elliptical basis bearing robust setae on anterior margin, posterodistal corner weakly lobate.



Figure 6. *Eoniphargus iwataorum* sp. nov., female holotype (NSMT-Cr 30782) **A** head, lateral view **B–D** dorsal margins of pleonites 1–3, dorsal views **E–G** epimeral plates 1–3, lateral views **H–J** dorsal margins of urosomites 1–3, dorsal views **K** antenna 1 (some flagellar articles are omitted), lateral view **L** antenna 2 (some flagellar articles are omitted), lateral view **M** upper lip, anterior view **N** right mandible, medial view **O** incisor and lacinia mobilis of right mandible, medial view **P** incisor and lacinia mobilis of left mandible, lateral view **Q** lower lip, anterior view **R** maxilla 1, dorsal view **S** apical robust setae on outer plate of maxilla 1, dorsal view **T** maxilla 2, dorsal view.

Coxal gills (Fig. 8D, E) ovate with stalks on gnathopod 2 and pereopods 3-6.

Pleopods 1–3 (Fig. 8F–I) with peduncles longer than rami, bearing distal seta on pleopods 1 and 2 and proximal seta on pleopod 3; retinacula paired (Fig. 8I); mediobasal margin of inner ramus with bifid plumose setae; rami well developed.



Figure 7. *Eoniphargus iwataorum* sp. nov., female holotype (NSMT-Cr 30782) **A** maxilliped, dorsal view **B** inner plate of maxilliped, dorsal view **C** outer plate of maxilliped, dorsal view **D** gnathopod 1, lateral view **E** palmar margin and dactylus of gnathopod 1, medial view **F** gnathopod 2, lateral view **G** palmar margin and dactylus of gnathopod 2, medial view **H** pereopod 3, lateral view **I** dactylus of pereopod 3, lateral view **J** pereopod 4, lateral view **K** pereopod 5, lateral view **L** pereopod 6, lateral view.


Figure 8. *Eoniphargus iwataorum* sp. nov., female holotype (NSMT-Cr 30782) **A**, **B** pereopod 7, lateral views **C** dactylus of pereopod 3, lateral view **D** coxal gill 4, lateral view **E** coxal gill 6, lateral view **F** peduncle of pleopod 1, lateral view **G** pleopod 2, medial view **H** peduncle of pleopod 3, lateral view **I** retinacula of pleopod 2, medial view **K** uropod 2, dorsal view **L** uropod 3, ventral view **M** telson, dorsal view.

Uropod 1 (Fig. 8J) with peduncle bearing three basofacial robust setae and dorsal robust setae; inner ramus length 0.7 times as long as peduncle, with two medial and one lateral robust setae, and ventroproximal seta; outer ramus length 0.9 times as long as inner ramus, with two robust setae on medial margin. Uropod 2 (Fig. 8K) with peduncle bearing dorsal robust setae; inner ramus as long as peduncle, bearing two medial robust setae and ventroproximal slender seta; outer ramus length 0.7 times as long as inner ramus, without marginal setae. Uropod 3 (Fig. 8L) with peduncle length 0.3

times as long as outer ramus, bearing robust setae on distal edge; inner ramus length 0.2 times as long as outer ramus, with seta on medial margin; outer ramus 2-articulate, proximal article with plumose setae on medial margin and robust setae on medial and lateral margins, terminal article length 0.2 times as long as proximal article with subapical setae.

Telson (Fig. 8M) length slightly shorter than wide, each lobe with two lateral and three apical robust setae and two dorsolateral penicillate setae, cleft for 67%.

Distribution and environment. The species is known only from its type locality in the Sabi River, Tochigi Prefecture, Japan.

Remarks. *Eoniphargus iwataorum* sp. nov. is similar to *E. kojimai* but differs from the latter in the following features (features of *E. kojimai* in parentheses): urosomite 3 without robust setae on dorsal margin (bearing robust setae), maxilla 2 with inner plate bearing seven setae in oblique row (nine setae), and uropod 2 without robust seta on lateral margin of outer ramus (bearing robust seta).

Eoniphargus kojimai Uéno, 1955

Figs 9–12 Japanese name: Kojima-chikayokoebi

Neoniphargus (Eoniphargus) kojimai Uéno, 1955: 148, figs 1–3. Eoniphargus kojimai: Straškraba, 1964, 138; Straškraba 1967, 127; Bousfield 1977, 301; Barnard and Barnard 1983, 581.

Material examined. \bigcirc 6.3 mm (NSMT-Cr 30785, G1905), \bigcirc 4.8 mm (NSMT-Cr 30786), Mamashita Spring (35.680066°N, 139.428283°E), Kunitachi, Tokyo, Japan, collected by K. Tomikawa on 25 June 2016. \bigcirc 5.0 mm (NSMT-Cr 30787, G 1930), \bigcirc 4.5 mm (NSMT-Cr 30788, G 1931), Hinochūōtoshokan Spring (35.655783°N, 139.382133°E), Hino, Tokyo, Japan, collected by K. Tomikawa on 25 June 2016.

Diagnosis. Urosomite 3 with dorsal robust setae. Epimeral plates 2–3 each with ventral robust seta. Peduncular article 1 of antenna 1 with robust seta on posterodistal corner. Antenna 2 with peduncular article 2 gland cone not exceeding end of article 3; calceoli present in male. Mandible with 5- or 6-dentate left incisor; 4- or 5-dentate left lacinia mobilis, right one bifid with many teeth. Inner plate of maxilla 1 with eight plumose setae. Inner plate of maxilla 2 with nine facial seta in oblique row. Peduncle of pleopod 3 without seta. Uropod 1 with peduncle bearing basofacial robust setae. Uropod 3 with inner ramus 0.25 times as long as outer ramus in female and 0.27 times in male; outer ramus 2-ariticulate, with plumose setae on medial margin. Telson length 0.9 times width, cleft for 69% of length.

Description. Female (NSMT-Cr 30785). Head (Fig. 9A) as long as pereonites 1 and 2 combined; eyes absent; inferior antennal sinus distinct with rounded angle. Dorsal margins of pleonites 1–3 (Fig. 9B–D) each with 4 setae. Posterodistal corners of epimeral plates 1–3 (Fig. 9E–G) weakly produced; ventral margin of epimeral plate 1 without setae, epimeral plates 2 and 3 each with ventral robust seta; posterior margins of epimeral



Figure 9. *Eoniphargus kojimai* Uéno, 1955, female (NSMT-Cr 30785) **A** head, lateral view **B–D** dorsal margins of pleonites 1–3, dorsal views **E–G** epimeral plates 1–3, lateral views **H–J** dorsal margins of urosomites 1–3, dorsal views **K** antenna 1 (some flagellar articles are omitted), lateral view **L** antenna 2 (some flagellar articles are omitted), medial view **M** upper lip, posterior view **N** right mandible, medial view **O** incisor and lacinia mobilis of left mandible, medial view **P** incisor and lacinia mobilis of right mandible, medial view **Q** lower lip, anterior view **R** maxilla 1, dorsal view **S** apical robust setae on outer plate of maxilla 1, dorsal view **T** maxilla 2, dorsal view.

plates 1–3 each with two setae. Dorsal margin of urosomite 1 (Fig. 9H) with slender setae and robust seta; urosomites 2 and 3 (Fig. 9I, J) with pair of dorsal robust setae.

Antenna 1 (Fig. 9K) length 0.22 times as long as body; length ratio of peduncular articles 1–3 in 1.0 : 0.8 : 0.4; posterodistal corner of peduncular article 1 with robust seta; accessory flagellum 4-articulate; primary flagellum 26-articulate. Antenna 2 (Fig. 9L) length 0.6 times as long as antenna 1; peduncular article 4 length 0.9 times as long as article 5; flagellum 13-articulate; calceoli absent.

Upper lip (Fig. 9M) with rounded apical margin bearing fine setae. Mandibles (Fig. 9N-P) with left and right incisors comprising five and six teeth, respectively; left lacinia mobilis comprising four teeth, right lacinia mobilis bifid with many teeth; molar process triturative with plumose seta; length ratio of palp articles 1-3 in 1.0: 2.3 : 1.7; palp article 1 without setae; palp article 2 with nine marginal setae; palp article 3 with pair of A-setae, several D-setae and three E-setae, lateral face with fine setae. Lower lip (Fig. 9Q) lacking inner lobes; outer lobes broad, shoulder rounded, with fine setae. Maxilla 1 (Fig. 9R, S) with subtriangular inner lobe, bearing eight plumose setae on medial margin; outer plate rectangular, with 11 serrate robust setae apically; palp 2-articulate, article 1 without setae, article 2 with three robust setae and slender plumose seta along apically to medial margins. Maxilla 2 (Fig. 9T) with inner plate bearing nine plumose setae in oblique row. Maxilliped (Fig. 10A-C) with inner plate exceeding end of palp article 1, subquadrate, bearing three subapical robust setae and medial robust seta; outer plate ovate, reaching middle of palp article 2, with row of robust setae along apical to medial margins; palp 4-articulate, article 2 longest with medial setae, nail of article 4 distinct.

Gnathopod 1 (Fig. 10D, E) with subquadrate coxa bearing setae along anterior to ventral margins; anterior and posterior margins of basis with long setae; carpus length 1.1 times as long as length of propodus and 1.7 times width of carpus; propodus length 1.9 times width, palmar margin weakly serrate, oblique, with three medial and two lateral robust setae; dactylus almost reaching posterodistal corner of propodus. Gnathopod 2 (Fig. 10F, G) with coxa expanded proximally bearing setae along anterior to ventral margins; posterior margin of basis with long setae; carpus length 1.2 times as long as length of propodus and 3.3 times width of carpus; propodus length 2.8 times width, palmar margin weakly serrate, almost vertical, with two medial and one lateral robust setae; dactylus reaching posterodistal corner of propodus.

Pereopod 3 (Fig. 10H, I) with subquadrate coxa, proximally expanded, bearing setae along anterior to ventral margins; anteroproximal and posterior margins of basis with long setae. Pereopod 4 (Fig. 10J) with coxa bearing setae along anterior to ventral margins, posteroproximally concave; anteroproximal and posterior margins of basis with long setae. Pereopod 5 (Fig. 10K) with bilobed coxa bearing seta on anterior and posterior lobes; anterior margin of basis with robust setae, posterodistal corner weakly lobate. Pereopod 6 (Fig. 11A) with bilobed coxa bearing two setae on posterior lobe; basis with robust setae on anterior margin, posterodistal corner weakly lobate. Pereopod 7 (Fig. 11B) with elliptical basis bearing robust setae on anterior margin, posterodistal corner weakly lobate.



Figure 10. *Eoniphargus kojimai* Uéno, 1955, female (NSMT-Cr 30785) **A** maxilliped, dorsal view **B** inner plate of maxilliped, dorsal view **C** outer plate of maxilliped, dorsal view **D** gnathopod 1, lateral view **E** palmar margin and dactylus of gnathopod 1, medial view **F** gnathopod 2, lateral view **G** palmar margin and dactylus of gnathopod 2, medial view **H** pereopod 3, lateral view **I** dactylus of pereopod 3, lateral view **J** pereopod 4, lateral view **K** pereopod 5, lateral view.



Figure 11. *Eoniphargus kojimai* Uéno, 1955, female (NSMT-Cr 30785) **A** pereopod 6, lateral view **B** pereopod 7, lateral views **C** pleopod 1, lateral view **D** retinacula of pleopod 1, lateral view **E** peduncle of pleopod 2, medial view **F** peduncle of pleopod 3 **G** coxal gill 4, lateral view **H** oostegite of pereopod 4, medial view **I** uropod 1, dorsal view **J** uropod 2, dorsal view **K** uropod 3, dorsal view **L** telson, dorsal view.

Coxal gills (Fig. 11G) ovate with stalks on gnathopod 2 and pereopods 3-6.

Pleopods 1–3 (Fig. 11C–F) with peduncles longer than rami, bearing distal seta on pleopod 1; retinacula paired (Fig. 11D); mediobasal margin of inner ramus with bifid plumose setae; rami well developed.



Figure 12. *Eoniphargus kojimai* Uéno, 1955, male (NSMT-Cr 30786) **A** antenna 1 (some flagellar articles are omitted), lateral view **B** antenna 2 (some flagellar articles are omitted), lateral view **C** calceolus on flagellar article of antenna 2, medial view **D** gnathopod 1, lateral view **E** palmar margin and dactylus of gnathopod 1, medial view **F** gnathopod 2, lateral view **G** palmar margin and dactylus of gnathopod 2, medial view.

Uropod 1 (Fig. 11I) with peduncle bearing three basofacial robust setae and dorsal robust setae; inner ramus length 0.6 times as long as peduncle, with two medial robust setae and two ventroproximal setae; outer ramus length 0.9 times as long as inner ramus, with two medial and one lateral robust setae. Uropod 2 (Fig. 11J) with peduncle bearing dorsal robust setae; inner ramus as long as peduncle, bearing two medial and one lateral robust setae; bearing two medial and one lateral robust setae; outer ramus length 0.7 times as long as inner ramus, with lateral robust setae; outer ramus length 0.7 times as long as inner ramus, with lateral robust seta. Uropod 3 (Fig. 11K) with peduncle length 0.3 times as long as outer ramus, bearing robust setae on distal edge; inner ramus length 0.3 times as long as outer ramus, with seta on medial margin; outer ramus 2-articulate, proximal article with plumose setae on medial margin and robust setae on medial and lateral margins, terminal article length 0.2 times as long as proximal article with subapical setae.

Telson (Fig. 11L) length slightly shorter than width, each lobe with two lateral and three apical robust setae, and two dorsolateral and 1 subapical penicillate setae, cleft for 69%.

Male (NSMT-Cr 30786). Antenna 1 (Fig. 12A) length 0.24 times as long as body length; accessory flagellum 3-articulate; primary flagellum 25-articulate. Antenna 2 (Fig. 12B) length 0.6 times as long as antenna 1; peduncular article 4 length 0.8 times as long as peduncular article 5; peduncular article 5 and flagellum with calceoli (Fig. 12C); flagellum 16-articulate. Gnathopod 1 (Fig. 12D, E) with carpus length 1.2 times as long as length of propodus and 1.6 times width of carpus; propodus length 1.5 times width. Gnathopod 2 (Fig. 12F, G) with carpus length 1.1 times as long as length of propodus and 2.3 times width of carpus; propodus length 2.2 times width, palmar margin with two medial and two lateral robust setae. Uropod 3 (Fig. 12H) with peduncle length 0.4 times as long as outer ramus, peduncle bearing bent robust setae on distal edge.

Distribution and environment. This species has been found in interstitial waters in Tokyo: the sand-filter bed of the Komae Purification Plant near the Tama River (Uéno 1955); two springs — Mamashita Spring, Kunitachi and Hinochūōtoshokan Spring, Hino (this study).

Remarks. The present specimens conform with the original description of *E. koji-mai* by Uéno (1955). However, our specimens differed from the original description in the left mandible with 4-dentate lacinia mobilis and maxilla 1 with 11 serrate robust setae on the outer plate, which was 6-dentate incisor and 5-dentate lacinia mobilis of the left mandible, and 10 serrate robust setae in Uéno's (1955) description. The exact number of these setae is difficult to ascertain because they are minute and overlap. This suggests that he may have misstated the number of setae. Unfortunately, the type specimen of this species is believed to be lost (Tomikawa et al. 2007), so the character could not be verified. Examination of the present material from Kunitachi and Hino, Tokyo revealed some features that were not mentioned in the original description: maxilla 2 with inner plate bearing nine plumose setae in oblique row, uropod 1 peduncle with facial robust setae, and maxilliped inner plate with three subapical robust setae and medial robust seta.

Key to species of Eoniphargus

1	Antennal sinus shallow; antenna 1 peduncular article 1 with slender seta on
	posterodistal corner; antenna 2 peduncular article 2 with elongate gland cone
	exceeding peduncular article 3; uropod 1 peduncle without basofacial robust
	setae; uropod 3 with uniarticulate outer ramus
	<i>E. glandulatus</i> Stock & Jo, 1990
_	Antennal sinus deep; antenna 1 peduncular article 1 with robust seta on pos-
	terodistal corner; antenna 2 peduncular article 2 with gland cone reaching
	distal end of peduncular article 3; uropod 1 peduncle with basofacial robust
	setae; uropod 3 with 2-articulate outer ramus
2	Right mandible with lacinia mobilis bearing 3 or 4 teeth; maxilla 1 with 6
	medial setae on inner plate
_	Right mandible with bifid lacinia mobilis bearing many teeth; maxilla 1 with
	8 medial setae on inner plate
	1

Molecular phylogenetic analyses

The obtained ML tree showed a topology identical to that of the BI tree (Fig. 13). The monophyly of Mesogammaridae was shown with the highest support (100% bootstrap support [BS] and 1.0 Bayesian posterior probability [BPP] in the ML and BI trees, respectively). Mesogammaridae formed a monophyletic group with two gammarids genera, *Barnardiorum* Iwan & Löbl, 2007 and *Rhipidogammarus* Stock, 1971 (BS = 99%, BPP = 1.0). Within Mesogammaridae, the first split formed *Mesogammarus* and (*Eoniphargus* + *Octopupilla*) (BS = 69%, BPP = 1.0). *Eoniphargus kojimai* is sister to *E. iwataorum* sp. nov. (BS = 76%, BPP = 1.0). The uncorrected pairwise distances among the three *Eoniphargus* species are 16.9–19.8% for COI and 1.3% for 28S.

Discussion

Although there are many taxonomic studies on the amphipods that occur in East Asian groundwaters (Akatsuka and Komai 1922; Derzhavin 1927; Uéno 1927, 1934), their species diversity is not fully understood. To date, studies on the amphipods in groundwater have been based on specimens from small streams in caves that are relatively easily accessible and incidentally collected from dug wells (Tomikawa et al 2008; Tomikawa and Nakano 2018). Recently, a variety of groundwater amphipods have been reported to occur in the interstitial waters of rivers and springs (e.g. Tomikawa et al. 2007), but little is known about their species diversity and evolutionary history.

In this study, we conducted a molecular phylogenetic analysis of Mesogammaridae, including one marine genus, *Mesogammarus*, and two subterranean genera, *Eoniphargus* and *Octopupilla*, and showed that they form a monophyletic group (Fig. 13). Our phylogenetic trees show that *Mesogammarus* was the first to diverge within Mesogammaridae, followed by *Octopupilla* and *Eoniphargus*. *Mesogammarus* occurs under coastal cobbles, *Octopupilla* in brackish and freshwater interstitial environments, and *Eoniphargus* only in freshwater groundwater (Tzvetkova 1965; Tomikawa et al. 2007). This suggests that species diversification in Mesogammaridae likely occurred through the adaptive dispersal of epigean marine ancestor into brackish and freshwater ground-water environments.

Mesogammaridae formed a monophyletic group with gammarid genera Barnardiorum and Rhipidogammarus. Barnardiorum occurs in epigean freshwaters in Tajikistan and Afghanistan, and Rhipidogammarus in brackish and freshwater groundwaters (interstitial waters) in the Mediterranean belt (Barnard and Barnard 1983;



Figure 13. Maximum likelihood tree for 1550 bp of nuclear 28S rRNA and mitochondrial cytochrome *c* oxidase subunit I markers. Numbers at nodes represent maximum likelihood bootstrap values and Bayesian posterior probabilities (Values below 60% for the former and 0.8 for the latter are omitted).

Özbek and Sket 2020). Hou et al. (2014) showed the Tethys Sea origin of Gammaridae and that geohistorical and environmental changes in the Tethys Sea facilitated the diversification of this group. Although the details of the evolutionary history of Mesogammaridae and related groups are not clear in this study, judging from the fact that their distribution extends from the North Pacific coast to Central Asia and the Mediterranean Sea, ancestral species that inhabited the coastal surface waters of the Tethys Sea may have diversified by adapting to different salinity and subterranean environments. *Barnardiorum* and *Rhipidogammarus* are presently treated as members of Gammaridae. Our results challenge their family affiliation, but the low statistical support for the results of the phylogenetic analyses in this study precluded further discussion of the monophyly of Gammaridae.

Prior to this study, two species of *Eoniphargus*, *E. kojimai* and *E. glandulatus*, were found in the interstitial waters in Japan and cave pools in the Korean Peninsula, respectively. In this study, we described two new species, E. iwataorum and E. toriii, found in the Kanto region of Japan, indicating that the species diversity of amphipods in interstitial water is higher than previously thought. Interestingly, *Eoniphargus* mainly occurs in interstitial waters and is rarely reported from the groundwater in caves. In contrast, the groundwater amphipod genus Pseudocrangonyx Akatsuka & Komai, 1922 is widely distributed in East Asian groundwater systems, and species of this genus often occur in cave groundwater (Lee and Min 2020). Speciation in subterranean environments is commonly a consequence of geographic isolation, as subterranean environments are always dark, oligotrophic, and ecological niches are narrow (Mammola and Isaia 2016; Mammola et al. 2018). Recently, however, the potential for diversification through niche differentiation has been demonstrated in Niphargus amphipods and Troglohyphantes spiders, even in energy-poor environments such as underground habitats (Fišer et al. 2013; Mammola et al. 2018). In Eoniphargus and *Pseudocrangonyx*, the choice between different groundwater environments, relatively open cave groundwater, and interstitial water may have played a role in the differentiation of the two genera.

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RESEARCH ARTICLE



Postembryonic development of the troglobitic planthopper species Valenciolenda fadaforesta Hoch & Sendra, 2021 (Hemiptera, Fulgoromorpha, Kinnaridae), with a key to nymphal instars

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Abstract

All nymphal instars of the recently discovered troglobitic planthopper species *Valenciolenda fadaforesta* Hoch & Sendra, 2021 are described. This represents the first documentation of the complete postembryonic development of any species in the family Kinnaridae. Characters of the external morphology are described and illustrated, and a key to the instars are provided to facilitate discrimination among the different nymphal stages. While *V. fadaforesta* nymphs share certain synapomorphies with other Fulgoromorpha (except the Tettigometridae), e.g., the cog-wheel structures of the metatrochanters, other characters may be correlated with the subterranean way of life of the species, and thus be autapomorphic, such as the absence of compound eyes in all instars.

Keywords

Caves, Iberian Peninsula, morphology, taxonomy, troglobite

Introduction

Fulgoromorpha is a large subgroup of the Hemiptera comprising at least 14,000 species in 21 extant and 15 extinct families (Bourgoin 2022). Despite their worldwide distribution (Bourgoin 2022), ecological diversity and economic importance (Denno and Perfect 1994), and many aspects of their biology are scarcely known. While postembryonic development has been studied in more detail in select Fulgoromorpha taxa (see e.g., Yang and Yeh 1994, and references therein, Emeljanov 1996; Asche 2015), information on nymphal morphology remains incomplete or lacking for most species. The only comprehensive treatments of Fulgoromorpha nymphs to date, including representatives of most taxa in family rank to date has been provided by Yang and Yeh (1994) and Emeljanov (2001). From their observations Yang and Yeh (1994) derived a compilation of characters considered to be distinctive of the 5th instar of Fulgoromorpha nymphs, and Emeljanov provided a hypothetical ground plan of certain aspects of nymphal morphology of Fulgoromorpha. One of the taxa which were neither covered by Yang and Yeh (1994) nor Emeljanov (2001) are the Kinnaridae. This family currently consists of 116 described species across 25 genera from the Palearctic, East Africa (partim), Arabian Peninsula and Oriental regions, as well as from the Nearctic and the Neotropics (Bourgoin 2022).

The nymphs of Kinnaridae have until recently been entirely unknown. This is most likely due to the lack of material, the reason for which may lie in their cryptic way of life. These apparently live in or close to the soil and/or subterranean crevices presumably feeding on roots (Fennah 1948, 1980). The nymphs of epigean species supposedly leave the nymphal habitat before or after the adult molt and feed and reproduce above ground, while adults of troglobitic species complete their life cycle underground. Cave-dwelling species of Kinnaridae are documented from Mexico (Fennah 1973), Jamaica (Fennah 1980), Brazil (Hoch and Ferreira 2013, 2016) and Spain (Hoch et al. 2021).

The first description of a kinnarid nymph (instar V) was given by Hoch et al. (2021) for the troglobitic *Valenciolenda fadaforesta* Hoch & Sendra, 2021 from Spain. Described from a cave in eastern karstic massif in the east of Iberian Mountain Range, in Valencia, Spain (the type locality), adults and nymphs were found to co-occur in the same habitat, thus offering the opportunity to obtain and study nymphs of all instars (Fig. 1).

Here we provide the first descriptions of the external morphology of instars I–IV of a representative of the Kinnaridae and summarize the results of Hoch et al. (2021). Our observations contribute to the knowledge of morphological alteration during nymphal development and help to complete the ground pattern of nymphal instars in Fulgoromorpha.



Figure 1. *Valenciolenda fadaforesta*, habitus of adult male (left) and instar nymph V (right). Body length of adult: 3.6 mm; body length of nymph: 2.9 mm. (Photo courtesy by Roberto García-Roa, www.robertogarciaroa.com).

Methods

Sampling

Immatures of *Valenciolenda fadaforesta* were located visually and by direct intuitive searching (DIS, sensu Wynne et al. 2019) within an undefined search radius, yet with special emphasis around root patches, in the deep cave zone at the type locality of the species: Murciélagos Cave, in Les Rodanes Municipal Natural Park which is located in Villamarchante, Valencia, Spain. Specimens were collected by hand during three visits to the cave, and immediately transferred to vials containing 70% ethanol (for dates and number of specimens see Table 1). As the present study focuses on the qualitative description of the nymphal instars of *V. fadaforesta*, we deliberately refrained from frequent visits and overcollecting because the cave in question is only one out of two localities where this species occurs. Since we do not have any knowledge about population size and life history of the species, we aimed at minimal impact to the environment and to the species. Measurements of body length were taken from freshly collected specimens using a Leica DMLS phase-contrast optical microscope with an ocular micrometre. For further examination specimens were washed with distilled water and then mounted on glass slides with Marc André II solution.

Table 1. Collection dates and number of nymphs per instar stage for each of the five instar stages, Murciélagos Cave, Les Rodanes Municipal Natural Park, Valencia, Spain.

Date	Instar I	Instar II	Instar III	Instar IV	Instar V	
11.09.2020	0	1	6	8	2	
18.10.2020	1	2	2	5	5	
28.11.2020	4	6	5	1	1	
Total	n = 5	n = 9	n = 13	n = 14	n = 8	n = 49

Morphological analysis

For morphological analysis and drawings, a Leica MZAPO stereoscopic microscope with *camera lucida* attachment was used. For medium-sized and larger specimens, the right metathoracic leg was removed and mounted separately, again with Marc André II solution. For scanning electronic microscopy, specimens were critical point-dried in a Tousimis Autosamdi 815, series A, coated with palladium-gold, and examined under Hitachi S-4100 and JEOL JSM-6335F (SEM).

Morphometric analysis

For morphometric analysis of leg proportions (length of femora and tibiae) we applied linear regression using measurements of the right metathoracic tibiae and femora from the legs mounted with Marc André II solution. We used OriginPro 8.0 software (Origin-Lab Corporation, Northampton, MA, USA) to test for suspected allometric growth.

Results

Assigning specimens to instars

Valenciolenda fadaforesta has five nymphal instars which coincide with the existing records for Fulgoromorpha. As overall body size varied widely among specimens studied and given the small sample size, it was not possible to discern nymphal instars based on this character alone. However, the linear regression analysis (length of femora/tibiae of the hind legs) revealed that our data points fell into 5 "groups" (Fig. 2). Upon closer morphological examination, we found that these groups coincided with the nymphal stages in other Fulgoromorpha as described by Emeljanov (2001). Separating the instars with certainty is thus possible on the basis of morphological characters (e.g., presence or absence of wing pads, shape and relative size of wing pads, etc.)

General diagnosis of nymphal stages

Valenciolenda fadaforesta nymphs are generally small, with body length, i.e. the distance between the anterior margin of the vertex to the caudal margin of the abdomen, ranging from 0.8 mm (1st instar, N1) to 3.2 mm (5th instar, N5). Bodily pigmentation is largely reduced, the integument is predominantly white; compound eyes and ocelli are absent, tegulae and wings are vestigial. The body is dorsoventrally depressed, longer than wide, with maximum width in the second thoracic segment at the base of the wing pads, maintaining approximate proportions in all nymphal stages. Frons, thoracic nota and abdominal segments IV–VIII covered by a variable number of sensory pit organs. The median tergite of the metathoracic segment (metathoracic postnotum) features a variable number of wax-secreting pores.



Figure 2. Regression analysis of metathoracic leg length in the nymphal instars of Valenciolenda fadaforesta.

Colouration: Vertex, frons, thoracic and abdominal nota, tibiae and tarsi yellowish-white; ventral regions of thorax and abdomen, coxae and femurs white; head and thorax carinae, rostrum and lateral teeth of metathoracic tibiae yellowish-brown.

Head: Vertex short, significantly wider than long medially. Frons narrow, ca. 1.45 times longer than maximally wide, with maximum width at the level of the antennae, devoid of median carina and provided with two submedial carinae that delimit a smooth area devoid of sensory pits. Frons separated from vertex by distinctly ridged margins. Between submedial carinae and lateral margins three rows of sensory pits that cover the section up to the frontoclypeal suture. Frontoclypeal suture almost straight, slightly curved in the medial section. Postclypeus and anteclypeus smooth, devoid of carinae. Elongated rostrum, well surpassing caudal margin of the metathoracic coxae by more than half its total length. Compound eyes absent. Antennae with 17 antennomers, scape short, ring-like, in some specimens not clearly visible, retracted into head capsule; pedicel subglobular, covered by plate organs (their number varying among instars) (Fig. 3); arista long and thin, composed by the 15 remaining antennomers. Third antennomer thickened, with a singular plate organ. Last antennomer slightly thickened, club-shaped (Fig. 3).



Figure 3. *Valenciolenda fadaforesta* nymphal structures (SEM) **A** V instar, base of antenna: scape, pedicel and antennomere III (dorsal view) **B** IV instar, cog-wheel structure at the trochanters of the hind legs, characteristic for all Fulgoromorpha (except the Tettigometridae) **C** III instar, distal antennomers **D** V instar, tip of the pretarsus, showing the claws and the arolia.

Thorax: Pronotum short, tricarinate, ca. 2.3 times wider than head at level below antennae. Median carinae dividing the pronotum into a small anterodorsal region, barely visible in dorsal view, and a triangular-shaped dorsal region endowed with a variable number of sensory pits depending on the nymphal stage. Mesonotum tricarinate and slightly wider than medially long. Median carinae separates mesothoracic postnotum from mesothoracic alinotum. Postnotum and alinotum furnished with a variable number of sensory pits. Alinotum with a variable number of longitudinal carinae depending on the nymphal stage and the degree of development of the wing pads. Tegulae vestigial. Metanotum slightly wider than medially long and tricarinate. Median carinae separates metathoracic postnotum from metathoracic alinotum. Metathoracic postnotum endowed with a variable number of waxsecreting pores depending on the nymphal stage. Metathoracic alinotum with an inconspicuous expansion on margins, vestige of the hind wing, and with a variable number of sensory pits. **Legs:** Trochanters of the hind legs medially with "cog-wheel"-like structure (as in nymphs of other Fulgoromorpha families, except Tettigometridae (Asche 1988)). This structure apparently facilitates the synchronization of hind legs during jumping (Burrows and Sutton 2013) (Fig. 3). Metathoracic coxae with meracanthus. Metathoracic tibiae laterally unarmed, with 7–8 slender teeth, arranged in a slightly concave row in its most distal region. Pretarsus with claws slender, short, not exceeding the arolium (Fig. 3). The proportions of meta-femur and meta-tibia undergo gradual modification in the course of consecutive molts: tibial length increases more strongly than femoral length, i.e. each instar display absolutely and relatively longer hind tibiae than the previous one (Fig. 2).

Abdomen: Ovoid, consisting of 9 segments, about as long as head and thorax together. Nota lacking carinae, mostly smooth except for small rows of tubercles on the lower margins of each segment. First two abdominal segments narrow, creating a distinct separation between thorax and abdomen. Tergites IV–VIII posses a variable number of sensory pits depending on the nymphal stage. Tergites IX–XI forming the genital complex, surrounded by tergite VIII. Dorsal unpaired process is likely the *anlage* of the anal style (segment X), framed laterally and ventrally by narrow, elongated lobes. The two short conical processes located medially at the level of the posteroventral corner of tergite IX could be interpreted as *anlagen* of either the genital styles (of the male), or of the gonocoxae VIII (of the female).

Key nymphal instars of Valenciolenda fadaforesta

1	Wings pads on mesonotum and metanotum absent2
_	Wing pads on mesonotum and metanotum present
2	Antennae provided with a single plate organ. Metanotum with two sensory
	pits and one pore on each side. Body length 0.8–0.9 mm
	Nymphal instar I (N1)
_	Antennae provided with three plate organs. Metanotum with two sensory pits
	and three pores on each side. Body length 1.3–1.4 mm
	Nymphal instar II (N2)
3	Metanotum with not fully developed wing pads, not reaching the distal end
	of the segment
_	Metanotum with fully developed wing pads, reaching the distal end of the
	segment. Antennae provided with ten plate organs. Metanotum with three
	visible sensory pits and fifteen pores on each side. Body length 2.8–3.2 mm.
	Nymphal instar V (N5)
4	Antennae provided with six plate organs. Metanotum with five sensory pits
	and seven pores on each side. Body length 1.5-1.7 mm
_	Antennae provided with seven sensory plate organs. Metanotum with six sen-
	sory pits and ten pores on each side. Body length 2.4–2.7 mm
	· · ·

Description of nymphal habitus per stage

Nymphal instar I (N1)

Small body, with a total body length between 0.8-0.9 mm (n=5). Antennae have a single plate organ located on the third antennomer and a subglobular pedicel devoid of plate organs. Tergites of the thoracic nota soft, weakly sclerotized, separated from each other by membranous area. Pronotum with 1 well-marked intermediate carina on each side of the body, separating the anterodorsal region of the pronotum, hardly visible from the dorsal perspective of the body, from the triangular dorsal region. Total absence of medial carinae in the mesonotum and metanotum that allows differentiating the tergites that make up these nota. Wing pads on meso- and metanotum absent. Sensory pits arranged in the thoracic nota as follows: pronotum with a total of 6 sensory pits on each side of the body, 3 located on the upper margin of the pronotum and 3 more central, parallel to the lower margin of the pronotum; mesonotum with 4 small sensory pits on each side, 2 located closer to the medial region of the segment and 2 in the lateral region; metanotum with a total of 2 larger sensory pits on each side, located in the lateral region of the segment. Metathoracic postnotum possesses a single pore on each side, parallel to the caudal margin of the segment. Tergites of the abdominal segments weakly sclerotized. Segments I-IV with a small row of tubercles on the lower margin, part of the cuticle sculpture. Sensory pits arranged in the abdominal segments as follows: segments IV-VII have a pair of sensory pits on both sides with each sensory pit located at the most distal ends of each segment. Segment VIII with a single small sensory pit on each side of the body. Segments IX–XI forming the genital complex (Fig. 4).

Nymphal instar II (N2)

Small body, with a total body length between 1.3–1.4 mm (n=9). Antennae with 3 plate organs, 2 on the pedicel and 1 on the third antennomer. Tergites of the thoracic nota with a greater degree of sclerotization, still quite separated from each other by membranous area. Pronotum with 1 well-marked intermediate carina on each side of the body, separating the anterodorsal region of the pronotum, hardly visible from the dorsal perspective of the organism, from the triangular dorsal region. Meso- and metanotum with 2 medial carinae on each side of the body dividing the segments in postnotum and alinotum. Wing pads on meso- and metanotum absent. Sensory pits arranged in the thoracic nota as follows: pronotum provided with a total of 6 sensory pits on each side of the body: 4 forming a row parallel to the lower margin of the pronotum features 6 sensory pits in total on each side of the body: 2 in the postnotum, parallel to the medial carina, and 4 grouped in pairs in the alinotum, one pair more central and the other in the lateral margin of the segment; metanotum with a total of 2 sensory pits located in the metathoracic alinotum. Metathoracic postnotum with 3 pores



Figure 4. *Valenciolenda fadaforesta* I–V nymphal instars. Top side left to right: nymphal instars I (N1), II (N2) and III (N3); bottom side left to right: nymphal instars IV (N4) and V (N5). Habitus in dorsal view.

on each side, parallel to the inferomedial margin of the metanotum. Tergites of the abdominal segments with a higher degree of sclerotization. Segments I–II smaller in size. Segments I–IV with a small row of tubercles on the lower margin, part of the cuticle sculpture. Sensory pits arranged in the abdominal segments as follows: segments IV–VII with a row of 3 sensory pits on each side of the body, located at the most distal ends of the segments. Segment VIII with a single small sensory pit on each side of the body. Segments IX–XI forming the genital complex (Fig. 4).

Nymphal instar III (N3)

Total body length between 1.5–1.7 mm (n=13). Antennae furnished with 6 plate organs, 5 on the pedicel and 1 on the third antennomer. Tergites of the thoracic nota well sclerotized, barely separated from each other. Pronotum with 1 well-marked intermediate carina on each side of the body, separating the anterodorsal region of the pronotum, hardly visible from the dorsal perspective of the body, from the triangular dorsal region. Meso- and metanotum with 2 medial carinae on each side of the body dividing the segments in postnotum and alinotum. Small wing pads present in the mesothoracic alinotum, with a longitudinal carina. Vestigial expansion of the hind wing pads in the metathoracic alinotum. Sensory pits arranged in the thoracic nota as follows: pronotum shows a total of 9 sensory pits on each side of the body, arranged in rows: 4 forming a row parallel to the upper margin of the pronotum and 5 forming another row parallel to the lower margin; mesonotum provided with a total of 11 sensory pits on each side of the body: 4 in the postnotum, forming a row parallel to the medial carina, and 7 in the alinotum (3 in the central region of the alinotum and 4 in the lateral margin following the longitudinal carina); metanotum with a total of 5 sensory pits located in the metathoracic alinotum: 2 parallel to the medial carina and 3 in the lateral region of the alinotum. Metathoracic postnotum with 7 pores on each side, irregularly arranged across notum, bilaterally and individually variable. Completely sclerotized abdominal segments. Segments I-II smaller in size. Segments I-IV with a small row of tubercles on the lower margin, part of the cuticle sculpture. Sensory pits arranged in the abdominal segments as follows: segments IV-VII with a row of 4 sensory organs on each side of the body, located at the most distal ends of the segments. Segment VIII with a single small sensory pit on each side of the body. Segments IX-XI forming the genital complex (Fig. 4).

Nymphal instar IV (N4)

Total body length between 2.4-2.7 mm (n=14). Antennae with 7 sensory plate organs, 6 on the pedicel and 1 on the third antennomer. Tergites of the thoracic nota well sclerotized, barely separated from each other. Pronotum with 1 well-marked intermediate carina on each side of the body, separating the anterodorsal region of the pronotum, hardly visible from the dorsal perspective of the body, from the triangular dorsal region. Meso- and metanotum with 2 medial carinae on each side of the body dividing the segments in postnotum and alinotum. Larger wing pads present in mesothoracic alinotum, provided with 2 longitudinal carinae. Vestigial expansion of the hind wing pads in the metathoracic alinotum. Sensory pits arranged in the thoracic nota as follows: pronotum features a total of 10 sensory pits on each side of the body, arranged in rows: 5 forming a row parallel to the superior margin of the pronotum and 5 forming another row parallel to the lower margin; mesonotum possesses 20 sensory pits in total, arranged on each side of the body: 6(5 + 1) in the postnotum, forming a row parallel to the medial carina, and 14 in the alinotum (3 in the central region of the alinotum, 4 forming a row parallel to the first longitudinal carina and 7 (4 + 3)forming two rows between the second longitudinal carina and the lateral margin of the mesonotum); metanotum with 6 sensory pits in total, located in the metathoracic alinotum: 3 grouped parallel to the medial carina and 3 forming a row in the lateral region of the alinotum. Metathoracic postnotum with 10 pores on each side, irregularly arranged across notum, bilaterally and individually variable. Completely sclerotized abdominal segments. Segments I-II smaller in size. Segments I-IV with a small row of tubercles on the lower margin, part of the cuticle sculpture. Sensory pits arranged in the abdominal segments as follows: segments IV–VII provided with a row of 5 sensory pits on each side of the body, located at the most distal ends of the segments. Segment VIII with 3 small sensory pits on each side of the body. Segments IX-XI forming the genital complex (Fig. 4).

Nymphal instar V (N5)

Thoroughly described by Hoch et al. (2021) with a supplementary description provided here. Total body length between 2.8–3.2 mm (n=8). Antennae furnished with 10 sensory plate organs, 9 in the pedicel and 1 in the third antennomer. Fully developed wing pads in the mesothoracic alinotum, with 2 longitudinal carinae. Metathoracic alinotum not visible, hidden under the mesothoracic wing pads. Metanotum with 3 visible sensory organs located in the metathoracic alinotum, parallel to the medial carina. Metathoracic postnotum with 15 pores on each side, irregularly arranged across notum. Completely sclerotized abdominal segments. Segments I–IV with a small row of tubercles on the lower margin, part of the cuticle sculpture (Fig. 4).

Discussion

The observable changes between the different nymphal stages of *Valenciolenda fadaforesta* consist of an increase in body size, an increase in the number of sensory pits and wax-secreting pores present in both the thorax and the abdomen, and the development of wing pads that develop into wings once they have reached maturity. The increase in length of the metathoracic tibiae and femora, as well as in the number of sensory pits and wax-secreting pores present in the tergites of the thoracic segments, have been the diagnostic characters used for the separation and classification of

the different nymphal stages. The structures studied in the cuticle of *V. fadaforesta* consist of sensory organs composed by wax filaments located in small pits, distributed throughout the thoracic region and in the caudal segments of the abdominal region, as well as small wax-secreting pores located in the postnotum of the metanotum. These individuals secreted a waxy substance that filled the cavities of the cuticle, made it difficult to study the structures and hide the pits where the wax filaments are located. So, it is possible that some of the smaller pits have gone unnoticed. The clear visibility of the wax-secreting pores present in the metathoracic postnotum has been very useful to differentiate between the smaller nymphal stages. It should be noted, however, that there exists some interindividual and intraindividual variability, with specimens that present a variable number of wax-secreting pores on both sides of the thorax (Fig. 5).

The life cycle of this species is unknown. Currently, we have no recorded evidence on the duration of single instars, overall lifespan, copulation, oviposition, or the eggs themselves. Furthermore, the literature on nymphal morphology and its development in the Kinnaridae family is also lacking, and the taxon has not been examined in the studies on nymphal morphology of the fulgoromorphs by Yang and Yeh (1994) and Emeljanov (2001) due to the lack of material.

The life cycle of *V. fadaforesta* is an hemimetabolous cycle composed of five nymphal stages and the adult stage. Juvenile stages of hemimetabolous insects resemble adults, except for the absence of functional wings and immature genital configuration. In these life cycles, embryogenesis gives rise to nymphs that possess adult characteristics.



Figure 5. Nymphal instar III of *Valenciolenda fadaforesta* (SEM). Thorax in dorsal view **A** specimen 1 **B** specimen 2. Note variation among individuals. In specimen 1 a waxy substance covers the tergites, absent in the specimen 2. The variation in the number of structures present in the same organism can also be observed in specimen 2, with 7 pores in the left section and 8 on the right.

Development of these nymphs consists of growth in size during which the growth ratio of the linear dimensions of the exoskeleton tends to remain constant between nymphto-nymph molts (Bellés 2020). Nymphs gradually develop into adult characteristics, with the final moult bringing forth the adult which has functional wings and fully developed external genitalia.

In V. fadaforesta the growth and development of the nymphal stages seems to follow a linear progression according to what can be observed (see Fig. 2). The increase of overall body length and proportions of femora/tibia length is more or less gradual from instar I – IV, while the change from instar IV –V is notably more distinct. Although we attempted to collect individuals across all possible sizes, these were small insects with tiny variations in size between different nymphal stages, which were difficult to discern with the naked eye. Apparently, overall body length alone is not sufficient to discriminate the nymphal stages, as it notably varies among individuals of the same instar. The observed variation may be due to genetic variation or may be due to individual modification during ontogeny (freshly hatched nymphs of a given instar are likely to be smaller than nymphs closer to the consecutive molt), or even caused by physiological condition (e.g., starved vs. well-fed). The cuticle in nymphs is not fully hardened, even in sclerites, and especially intersegmental membranes are rather extensible. The correct nymphal stage can ultimately only be determined upon examination of cuticle structures and measurement of the legs. The base pattern of the fulgoromorphan Hemiptera consists of five nymphal stages, with the only exception of Lycorma delicatula (White, 1845) (Fulgoridae) which has only four (Dara et al. 2015). Our observations on the nymphal development of V. fadaforesta are consistent with the existing literature on this Hemiptera taxon (Liang 2001; Hoch and Ferreira 2016; Hoch et al. 2021) and other closely related taxa (Wilson and Tsai 1982; Chen and Yang 1995; McPherson and Wilson 1995).

In the samples examined, individuals belonging to the same nymphal stage can be observed showing great differences in the body size and size of the abdominal region. When observed under a stereomicroscope, some individuals had a more "swollen" abdomen, although the size of the thorax and the cuticle that covers the abdominal tergites maintained its proportions (Fig. 6). At first glance, this variation in the size of the abdomen could result in a misclassification of the nymphal stages. Thus, measurement of its legs and the confirmation of key structures in its cuticles (i.e., presence or absence of wing pads, relative size of wing pads, number of sensory plate organs on the pedicel, number and distribution of sensory pit organs on pronotum, mesonotum and metanotum and number and distribution of wax-secreting pores) are required.

Regarding the size of nymphs, there was some variation of overall body size among individuals of the same nymphal instar, perhaps due to physiological conditions and individual development. Phenotypic variation in growth rates was present in all living organisms, and their plasticity was affected by multiple environmental variables where temperature and food availability were most influential (Lee and Roh 2010). Regarding temperature, underground habitats are one of the few natural systems where microcli-



Figure 6. Body size variation in nymphal instars I–V (stereomicroscope photograph). Nymphal instar V are largest but display high degree of variation in overall abdomen size (see text for possible explanation).

matic conditions can reach levels of homogeneity otherwise only achieved under laboratory conditions. The stable conditions in the deep cave zone thus rule out temperature oscillations as the determining factor underlying the observed phenotypic variation among individual nymphs of the same instar, e.g., in total body size (Sánchez-Fernández et al. 2018). The section of the cave where most of the nymphs were collected, the so-called "Root Room" (or *Sala de las Raíces*) in Murciélagos Cave, is no exception, with little variable temperatures between 16.5 °C and 18.3 °C throughout the year (Sendra et al. 2015). The variations in the size of the collected individuals could then be explained by differences in the stage of development or by differential access to the food source.

The gradual increase of absolute and especially relative length of the hind-tibia in consecutive instars may be indicative of increasing mobility in the course of nymphal development. Field observations of another obligately cavernicolous planthopper species, although in a different taxon of family rank, *Oliarus polyphemus* (Cixiidae) from lava tubes on Hawaii Island, revealed that earlier instars (I–III/IV) appear to be rather sedentary, sitting on roots and feeding. Older instars however, especially instar V, show dynamic mobility patterns, actively moving away from roots, most likely in search of a secure place suitable for molting into adults (Hoch, personal observation; also see Hoch and Howarth 1993). It is conceivable that nymphs of *Valenciolenda fadaforesta* show similar mobility patterns.

To discern the sexes of immatures is challenging. Nymphal instars I–IV do not present sexual structures that allow clear differentiation between male and female specimens. In the fifth nymphal stage, two different genital phenotypes exist: in some, yet not all, V instar nymphs we found two inconspicuous conical processes in the posteroventral region of segment IX (Fig. 7). These were only detectable under high magnification (SEM), and could neither be seen with the naked eye or even with the use of a stereomicroscope. These conical processes have been interpreted by Hoch et al. (2021) to be either precursors of the genital styles of the male or the gonocoxae VIII of the female. Sexing immatures of *Valenciolenda fadaforesta* is further impeded by the fact that highly modified external female genitalia are characteristic for the Kinnaridae (as well as the closely related taxon Meenoplidae), and even females of epigean species display strongly reduced ovipositors (Wilson 1983).



Figure 7. Nymphal instar V of *Valenciolenda fadaforesta* (SEM). Tip of abdomen in dorsodistal view. Conical processes (**A**, arrow) located medially at the level of the posterioventral corner of tergite IX present in **A** and absent in **B**.

Conclusions

The results and observations presented here are based exclusively on field samples. These first ever descriptions of all nymphal instars of a species of the Kinnaridae add to the existing knowledge of postembryonic development in this taxon, and further contribute to the reconstruction of ground pattern, or body plan, of Fulgoromorpha nymphs. It should be kept in mind, however, that the study organism, Valenciolenda fadaforesta, is an obligate troglobiont, and thus a highly specialized species. Certain morphological configurations may have evolved as reductive characters and may thus represent autapomorphies for this particular species rather than for the entire family. Since Valenciolenda fadaforesta is the only representative of the Kinnaridae of which nymphs have been available for study, a comparison with nymphs of epigean Kinnaridae species has not been possible. Accordingly, we cannot with certainty distinguish between potential autapomorphies for the species and those for the entire lineage. Suspected autapomorphies for V. fadaforesta are the absence of compound eyes in all instars, and perhaps related to it, the extremely short and wide vertex. Similar alterations of head capsule architecture have been observed in other cavernicolous Fulgoromorpha. Hoch and Howarth (1989), in a study on cavernicolous Cixiidae from Australia, hypothesized that "the broadening of the vertex [...] is apparently correlated with the reduction of compound eyes, and might have resulted from mechanical competition within the head capsule during morphogenesis, analogous to that which Berger and Howard (1968) reported for birds".

Given its occurrence in a specialized habitat yet in a comparatively easily accessible location, in the vicinity of academic facilities, *Valenciolenda fadaforesta* is a potential model organism to study morphogenesis in an obligately cavernicolous insect. In order to develop a protocol for rearing *Valenciolenda fadaforesta* in the laboratory, further studies are required to understand life history, longevity, reproduction rate, and host associations as well as to determine environmental factors controlling population size. Information on these will also be pivotal in developing strategies for management and conservation of the caves and other organisms inhabiting them.

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RESEARCH ARTICLE



Behavioral observations of the olm (Proteus anguinus) in a karst spring via direct observations and camera trapping

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Abstract

The olm (*Proteus anguinus*), an endemic amphibian of the Dinarides' underground waters (Europe), is one of the world's most widely known subterranean species. Although various aspects of olm biology have been extensively studied, the data on their behavior in the wild remain scarce mostly due to inaccessibility of their natural habitat. Yet, olms also occur in several karstic springs during nighttime. These are easier to access and present an exciting opportunity to study olm behavior in nature. Here, we report on systematic observations of olms in one such spring in Slovenia, where we observed them for nine consecutive summer nights, coupling direct on-site observations with IR camera trap recordings. We used IR camera trap recordings to construct simple ethograms, as well as to quantify olm movement activity by video-tracking. Olms regularly occurred on the surface during the night, and dawn appeared to be a key stimulus for their retreat underground. They were constantly active, but rarely swam far from the spring. Despite the short-term nature of the study, we collected new occurrence and movement data, and at the same time tested the usability of IR cameras for surveying olm presence and behavior in nature. Experience gained through the study may prompt long-term and more complex behavioral studies using similar approaches.

Keywords

Behavioral conservation, cave salamander, co-occurrence, ethogram, habitat choice, IR cameras, movement activity, remote sensing, video-tracking

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Introduction

The olm (*Proteus anguinus* Laurenti, 1768) is one of the most charismatic and widely known subterranean species, with a more than 300 years long history of research (Aljančič 2019). It is an endemic of the underground waters of the Dinarides, distributed from Italy on the northwest to Bosnia and Herzegovina and Montenegro on the southeast (Sket 1997; Gorički et al. 2017), with over 500 localities known to date (SubBioDB 2022). The olm is classified as a vulnerable species on the IUCN Red List (Arntzen et al. 2009), protected by several national legislations, and listed in Annex II of the Habitats Directive (The Council of European Communities 1992). Its populations are at risk throughout its distribution range with the main threats being habitat destruction by hydroengineering and construction (Koller Šarić 2019), as well as agricultural and industrial water pollution (Bressi 2004; Hudoklin 2011). More locally the olm's populations are threatened also by the presence of toxins in the environment and even poaching (Pezdirc et al. 2011; Năpăruş-Aljančič et al. 2017). Environmental conditions at many of the olm's localities remain unfavorable (Sket 1997; Hudoklin 2011), calling for an urgent development and implementation of conservation plans.

Over the last decades, the emergence of new tools and methods in the conservation biology already provided first steps towards its effective conservation. For example, molecular tools were developed and used to assess the genetic structure of olm populations (Gorički and Trontelj 2006; Zakšek et al. 2018; Vörös et al. 2019), providing a basis for future identification of sites and populations of the highest conservation priority. Further, the application of environmental DNA approaches facilitated the detection of olm presence and supplemented its previously known geographical distribution (Gorički et al. 2017; Vörös et al. 2017). Significant progress in olm's developmental biology, essential for sustainable captive breeding has been made in the last years (Gredar et al. 2019), and even sanctuaries for injured animals and individuals washed-out from their subterranean habitat were established (Aljančič et al. 2014; Lewarne 2018). Finally, deciphering the olm's genome is underway, promising great potential for further progress in olm conservation (Kostanjšek et al. 2022). Nevertheless, assessing the status of olm populations remains challenging (Trontelj and Zakšek 2016), largely due to habitat inaccessibility and incomplete knowledge on the biology, life history, ecology, and behavior of this species.

Most of the current knowledge on olm biology is based on observations in the laboratory or semi-natural conditions. Yet, the success of conservation actions heavily depends on our understanding of species' behavior in their natural habitats (Caro 1999; Blumstein and Fernandez-Juricic 2010). As observing olms in their natural habitats is difficult and commonly requires cave diving performed by specially trained researchers using expensive equipment, such knowledge is scarce. The most comprehensive work on olm behavior in the natural habitat has been done by Briegleb (1962) and the most recent finding of this approach is that olms show extremely high site-fidelity (Balázs et al. 2020). On the other hand, easily accessible sites, such as karst springs, offer a unique opportunity for *in-situ* behavioral observations of otherwise inaccessible populations using relatively cheap equipment. Although most records of olms in epigean habitats are only accidental, they occur in several perennial springs during nighttime (Sket and Arntzen 1994; Bressi et al. 1999; Bressi 2004; Kordiš 2016). However, there is currently no data on the regularity or fraction of olm's populations occurring in springs, and no systematic observations were conducted at such sites so far.

To complement the existing knowledge on the olm occurrence and behavior in karst springs, we conducted a nine-day systematic survey during nighttime in a spring in southeastern Slovenia. Besides direct on-site observations, we employed infrared (IR) cameras for indirect observations of olms. The importance of IR cameras for detecting remote wildlife and rare species has been widely acknowledged (Cutler and Swann 1999; Swann and Perkins 2014). IR cameras are often used to follow the presence and abundance of species and to study different aspects of animal behavior in their natural environments. As such, they are increasingly important in wildlife monitoring and conservation (Swann and Perkins 2014).

Thus far, IR cameras have been used to observe olms in the laboratory or under semi-natural conditions, as well as in the natural cave habitat (Balázs and Lewarne 2017; Lewarne 2018). However, IR cameras have not been used before to monitor olms in karst springs. In the present study, we aimed to evaluate whether the usage of IR cameras enables acquiring reliable data on olm behavior. Additionally, we explored possibilities to analyze the recordings from IR cameras to obtain quantitative data on olm presence and movement in the epigean part of the spring during nighttime.

Materials and methods

Site description

We observed the olms in a perennial karst spring located in southeast Slovenia. Due to potential threats to the population and internal research policy in Slovenia, we do not provide exact name and location of the spring.

Groundwater surfaces from two main springs and fills a shallow pool (hereafter referred to as "surface pool"; Fig. 1A) which continues as a small brook after approximately 10 meters. The bottom of the pool is covered mostly with fine gravel intermixed with silt, and a few larger rocks. Treetops in the immediate surroundings shade the pool throughout the day.

The water level of the surface pool fluctuates seasonally. In summer it is the lowest and thus with the best visibility, making summertime the most appropriate season for observing olms at this location. The surface pool was approximately 20 cm deep at the time of our observations. Visibility remained the same throughout all days of observations and was not affected by rainfall. The temperature in the spring and surface pool was constant, 11.5 ± 0.5 °C.



Figure 1. A ground plan of the karst spring in which olms (*Proteus anguinus*) were observed **B** an example of trajectories of three olms (different colors correspond to different individuals) during night 8, obtained by processing the images from the IR camera 1.
Fieldwork and camera setting

We observed occurrence and behavior of olms in the surface pool for nine consecutive nights, between 17th and 27th July 2019. For the first five nights, we observed olms directly on-site, while for the last four nights we coupled the direct observations with infrared (IR) camera recordings.

We carried out the direct on-site observations in three time periods per night. The first visit started at sunset (approx. 20:40 Central European Summer Time (CEST)) and lasted 1–3 hours. We returned to the spring after midnight (approx. between 00:00 and 2:00 CEST) for 20–30 minutes. The third and last visit was in the morning, one hour before sunrise (approx. 5:30 CEST). We provide exact observation times for each night in Suppl. material 1. During the direct on-site observations, we illuminated the surface pool every 10 minutes, counted the olms, and noted their position. To avoid disturbing the olms with too bright lights, we used only brief illumination time and dimmed red LED lights to which they appear to be less responsive according to observations of captive specimens (Schlegel et al. 2009).

Two IR cameras (Maginon WK 4HDW) were positioned at the opposing banks of the surface pool to cover an area as large as possible (Fig. 1A). The cameras were set to capture one frame per minute, starting one hour after sunset and finishing at sunrise. In total, direct observations lasted for 2–3 hours per night, while cameras were set to record for 7–8 hours per night.

Using both observation methods, we identified the holes through which olms emerged to the surface or retreated underground (hereafter referred to as "surfacesubterranean corridors") and determined the number of olms simultaneously present in the surface pool. For each olm we also noted its first and last occurrence in the surface pool during the night. The methods used did not enable reliable distinction of individual olms between nights.

Analysis of images recorded with IR cameras

We used images recorded with IR cameras to construct simple ethograms for each olm for the time of its presence in the surface pool. Although image quality did not allow recognition of complex behaviors, such as e.g., food searching, feeding, and agonistic behavior, we could clearly define and quantify the duration of four basic behaviors related to olms' position in the spring, named "emerging", "outside", "retreating" and "inside", which are described in detail in Table 1. The cameras covered the entire olms' movement in all but one case, when one olm swam further downstream. This event was treated as behavior "outside" until the animal reached the field of view again.

In addition, we used the acquired images to quantify olms' movement activity by video-tracking analysis. With both IR cameras, we captured 2713 images in total. Due to camera lens fogging or people in front of the camera, we discarded 424 (16%) images. We then carefully checked the remaining 2289 (84%) images for the presence of olms and detected them on 996 (37%) images. Due to the low quality of the raw images, we manually marked individual animals on these images by adding a 45-pixel

colored circle on olms' heads in Adobe Photoshop CS6. To distinguish between individuals, we used circles of different colors.

Next, we converted the modified image sequences to videos and performed videotracking in Bonsai 2.5.2. (Lopes et al. 2015) (Fig. 1B). We tracked the olms for the whole time that their heads were visible in the surface pool, disregarding the previously defined behaviors (Table 1). We retrieved the coordinates of the olms' positions from each camera separately, from which we calculated the total path covered and movement speed. We report video-tracking results from images obtained by camera 1 (Fig. 1A), as it better captured the olms' movement around the surface pool and provided more data. We used Pearson's correlation coefficient to assess the relationship between time spent in the surface pool and total path covered. Since shooting-angle of cameras prevented accurate distance measurements, we expressed the covered distances and movement speed using olm body length (bl) as a relative distance unit, or as an approximation of metric units. For the relative distance unit, we measured the olms' length in pixels multiple times (3-5 repeated measurements) in up to six images where they were well visible and used mean value in all analyses. For approximation in metric units, we assumed that the body length of the olms ranged between 15 and 25 cm. Lastly, when multiple individuals were simultaneously present in the surface pool, we analyzed their interactions by quantifying their mutual distance. We analyzed four pairs of co-occurring olms (one from night 6 and three from night 8) and measured the distance between their heads using body length of the larger olm in the pair as a relative distance measure.

The methods used did not enable reliable distinction of individual olms between different nights. Thus, results presented in Table 3 and Fig. 3 might be replicated observations of the same individuals during different nights. While we cannot completely exclude the chance of misidentifying the olms during same night, we argue that distinction of individuals based on their positions and body size was reliable.

Video-tracking data analyses and result visualizations were carried out in R 4.0.3 (R Development Core Team 2022) using the packages ggplot2 (Wickham 2016), dplyr (Wickham et al. 2019), scales (Wickham and Seidel 2020), and ggpubr (Kassambara 2020).

Table 1. Description of the four basic behaviors used to construct simple ethograms and quantify the olms' presence in the surface pool.

Behavior	Description
emerging	The animal is emerging from the subterranean part of the spring to the surface pool. The behavior starts when the head of
	the animal first appears from the subterranean corridor and stops when the whole animal emerges to the surface pool or
	retreats underground.
outside	The whole animal is present in the surface pool. The animal is still or moving around the pool.
retreating	The animal is retreating from the surface pool to the subterranean part of the spring. The behavior starts when the head
	of the animal first disappears into the subterranean corridor and stops when the whole animal retreats underground or
	returns to the surface pool.
inside	The animal is not present in the surface pool, the whole body is underground.

Results

Olm presence in the spring

During the first five nights, when olms were observed only directly, their presence in the surface pool was recorded on three nights. Their number was either one or at most two simultaneously present olms in the surface pool. During the next four nights, when we observed the olms also via IR cameras, we recorded up to three simultaneously present olms in the surface pool (Table 2).

Via direct observations, we identified two surface-subterranean corridors that olms used to transition from underground to the surface or vice versa. An additional, third corridor was discovered from IR cameras recordings. Olms first emerged in the surface pool one hour after sunset (Fig. 2). First emergences were equally distributed across almost four consecutive hours, i.e., from approximately 21:30 to 1:00 CEST (approx. 1-4.5 hours after sunset). On the other hand, last retreats of olms to the underground mostly clustered around 5:00 CEST (approx. 0.5-1 hour before sunrise). In three observations, animals retreated distinctively earlier (Fig. 2). Both the timing of first emergence and last retreat suggest sunlight as the most probable factor the olms use to guide their presence in the surface pool. At the time of their first emergence, at approx. 21:30, we had to use red LED lights to illuminate the surface pool, while in the morning, from approx. 4:30, we were able to observe olms without any additional lights. Connecting the first emergence and last retreat timing of the same individuals (grey lines in Fig. 2) showed considerable variation in the timing of olm presence in the surface pool. With more data at hand and ideally with known individual identity, such analysis could potentially test the presence of distinct behavioral types, e.g., "early bird" and "night owl" chronotypes or shyer and bolder individuals.

Behavior and movement activity of olms

We used IR camera recordings from three nights to quantitatively describe the behavior and movement activity of olms in the surface pool. We excluded the seventh night from the analyses due to the camera lens fogging and consequent unreliable detection of olms.

Night	Observation method	Number of olms observed
1	Direct	1
2	Direct	0
3	Direct	0
4	Direct	2
5	Direct	2
6	Direct and IR cameras	3
7	Direct and IR cameras	3
8	Direct and IR cameras	3
9	Direct and IR cameras	1

Table 2. Summary of observation methods and number of olms observed in the surface pool for each night.



Figure 2. Observations of the first emergence (green dots) and last retreat (orange dots) of olms in the surface pool based on direct and IR camera observations. Grey lines connect the first emergence and last retreat time of the same animal within one night.

Table 3. Total duration of behaviors, path covered, and movement speeds for individual olms. The individual ID numbers (Ind.) correspond to those in Fig. 3. Note that numbers 1–7 represent the number of observations and do not imply the number of individuals, as we could not reliably distinguish them between different nights. Path and speed are given in body length (bl) units.

		Beh	avior duration		Path covered [bl]	S	peed [bl/min]	
Night	Ind.	Emerging	Outside	Retreating		Minimum	Average	Maximum
6	1	NA	8 min	NA	2.4	0.04	0.34	0.68
6	2	13 min	1 h 41 min	1 min	37.3	0.01	0.30	2.28
6	3	14 min	3 h 21 min	18 min	87.1	0.01	0.36	1.42
8	4	50 min	2 h 5 min	3 min	36.7	0	0.22	1.10
8	5	4 min	37 min	2 min	17.1	0.03	0.38	1.28
8	6	22 min	4 h 19 min	19 min	104.2	0	0.34	2.24
9	7	14 min	2 h 47 min	1 min	66.1	0.004	0.36	1.69
	$Mean \pm SD$	$20 \pm 16 \min$	$128\pm89~min$	$7 \pm 9 \min$	50 ± 37	0.01 ± 0.01	0.33 ± 0.05	1.53 ± 0.59

We analyzed the behavior of three olms on the first two nights and a single olm on the last night, resulting in a maximum of seven olms. Nevertheless, the actual number of animals in the study might as well be lower, due to our inability to reliably discriminate the individuals occurring on different nights. Most olms were active for several hours during the night. On average, they took almost three times longer to appear from the underground ("emerging" behavior) than to retreat underground from the surface pool ("retreating" behavior) (Table 3, Fig. 3). This indicates that the olms are likely more cautious when emerging to the surface from their primary subterranean habitat. Five out of seven olms emerged to the surface and retreated underground several times during the night before they finally retreated at sunrise (Fig. 3). The total path covered by animals strongly positively correlated with the duration of their presence in the surface pool: the olms which were outside for longer also covered



Figure 3. Simple ethograms of the seven olms observed in the karst spring with IR cameras. Individual olms are marked with numbers which correspond to those in Table 3. Note that numbers 1–7 represent the number of observations and do not imply the number of individuals, as we could not reliably distinguish them between different nights.

the longest path ($\rho = 0.986$, p < 0.001) (Table 3). Assuming a body length of 15 and 25 cm, paths ranged from 0.4 m to 15.6 m (mean ± SD: 7.5 ± 5.6 m) and from 0.6 m to 26.1 m (mean ± SD: 12.5 ± 9.3), respectively.

Average moving speeds were similar among all olms, with a mean of 0.33 ± 0.05 bl/min and a range of 0.22-0.38 bl/min. On the other hand, olms differed in their maximum speeds, with a mean of 1.53 ± 0.59 bl/min and ranged from 0.68 to over 2.2 bl/min. Interestingly, their minimum speeds were only rarely zero, implying a rather constant movement within the surface pool after their emergence to the surface. Assuming body lengths of 15 cm and 25 cm, their average speed was 4.9 ± 0.8 cm/min and 8.2 ± 1.3 cm/min, respectively. Their maximum speed was 22.9 ± 8.8 cm/min (assuming 15 cm body length) and 38.2 ± 14.7 cm/min (assuming 25 cm body length).

Co-occurrence and interaction of olms

We observed four pairs of olms simultaneously present in the spring: pair 1 (individuals 2 and 3) during night 6, and pair 2 (individuals 5 and 6), pair 3 (individuals 4 and 5), pair 4 (individuals 4 and 6) during night 8 (Fig. 3). The duration of individual co-occurrence differed between pairs. It was roughly 20 minutes in three pairs, but it



Figure 4. Four cases of an interaction between a pair of olms co-occurring in the surface pool. Individual olm's numbers match those in Fig. 3. The plotted distance is the distance between both olms' heads and expressed in body lengths [bl] of the larger olm in the pair. One body length distance is indicated with a dashed line. Time 0 represents the start of co-occurrence, i.e., the moment when the second olm of the pair emerged to the surface pool while the first was already present.

lasted almost 2.5 hours in one pair (Figs 3 and 4). Most of the time, the olms were much more than one body length distance apart, and when they came close, they soon moved away from each other again (Fig. 4). This suggests that when multiple olms are present in the surface pool, they might avoid each other.

Discussion

The combination of direct and indirect observations of olms provided valuable data on their occurrence and movement activity in a karst spring and associated surface pool. By conducting the first systematic observation of olms and analysis of their behavior in a spring, we complement the existing knowledge of this enigmatic species in its natural habitat. Olm behavior in their natural environment is an important, but unfortunately rarely studied aspect of the species' biology and Briegleb's (1962) classic work remains the most comprehensive source of relevant information. Karst springs may provide an opportunity to further explore possibilities for assessing olm behavior and collecting behavioral data. Below, we summarize our observations and discuss potential challenges, improvements, and advantages of the methods used.

Our observations indicate that olms regularly occur in the epigean habitat at night during the summer, and that sunlight represents the most likely stimulus for olms to retrieve to the subterranean habitats. The reasons for the occurrence of olms in karst springs are not yet clear. The springs may simply serve as an extension of the olms' primary habitat during the night. The emergence of olm at the surface could simply be the result of their nocturnal movement along the subterranean-surface corridors when both habitats are not clearly divided by sunlight. However, some evidence suggests deliberate presence on the surface. First, olms quickly evade the potential threats (e.g., strong light and water disturbance), by swimming directly to holes leading underground (unpublished personal observations at several sites), suggesting exceptional orientation and/or spatial memory. Second, it has been suggested that olms emerge to the surface to feed in a food-rich environment, a prediction supported by a few observations (Sket and Arntzen 1994; Bressi et al. 1999; Recknagel et al. 2022). Olms are apex predators underground (Briegleb 1962), but might become a prey of larger epigean animals in or close to epigean habitats. This might further explain their movement in the close vicinity of the surface-subterranean corridors and retreat at sunlight.

In the future, several other aspects of the olm behavior might be addressed using the same observation methods, such as seasonality and interactions between animals. We observed the olms only in summer. If sunlight is the only factor keeping them underground, they should spend a relatively larger amount of time on the surface in the fall and winter when nights are long, and days are short. However, if they emerge to the surface mainly to feed, we might expect to see this behavior more often in parts of the year when food is usually more abundant at the surface or when food is particularly scarce underground. Our dataset did not allow a thorough analysis of olm interactions, but we were still able to detect and distinct four events of a co-occurring pair of olms. Most of the time, the two olms were much more than a body length apart and quickly moved away from each other when they got closer. To determine if they are actually avoiding each other, exhibit territorial behavior, or perhaps compete for food, more data and further studies are needed.

Compared to direct on-site observations, those via IR cameras provided more data on olms' emergence to and retreat from the surface, the use of surface-subterranean corridors, and their movement activity. At the same time, direct observations were valuable as notes on olm positions within the spring enabled easier identification of the animals on camera images. Our results show that the images captured by IR cameras are useful for detection and monitoring of olms and can be further analyzed to obtain behavioral data. For the first time, we have extended the use of IR camera recordings of olms beyond descriptive results by providing quantitative data on their movement. On the other hand, there are some limitations associated with the extraction of the data from raw IR camera images. First, we only tested the cameras in good weather conditions, and cannot provide any information about their usability in bad weather, e.g., fog or rain. Second, the images required additional processing to obtain movement and behavior data due to poor contrast between olms and the background. Our approach to this issue was relatively simple, yet probably too time-consuming for manually processing images in longer behavioral studies. There are several possible solutions for such cases, including better IR illumination, optimized camera positions (e.g., from above rather than from the side) and using more complex methods to process the images. Despite some limitations, we conclude that IR camera recordings are reliable and appropriate for the extraction of both qualitative and quantitative data.

In the future, our approach could be improved by placing more cameras to cover as wide an area as possible. It could further be improved by employing remote-controlled IR cameras capable of remote live streaming. These would avoid possible disturbances caused by our presence at the study site, which might have affected the olms' behavior. Wider coverage and no direct disturbance combined with accurate distinction of individual olms would allow the detection and recognition of more complex behaviors and consequently the construction of more detailed and informative ethograms. This would enable more in-depth studies on olm movement and use of epigean space, feeding, and predator-prey related behaviors, as well as studies on intraspecific interactions – all of which are key behavioral domains to consider in conservation efforts (Berger-Tal et al. 2011). Lastly, well-placed IR cameras could help detecting and monitoring harmful human activities like disturbance and even poaching at olm localities. We conclude that studies such as ours, although short-term and small-scale, represent a step toward integrating knowledge on olm behavior into plans of its conservation.

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Supplementary material I

Figure S1

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Data type: Png image.

- Explanation note: Overview of the methods used and related observation durations for all nine nights of the study.
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RESEARCH ARTICLE



A new species of Allobathynella (Crustacea, Bathynellacea, Parabathynellidae) from the hyporheic zone of the Hangang River, South Korea

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Abstract

Bathynellacea including the parabathynellid genus *Allobathynella* Morimoto & Miura, 1957 is commonly found across the subterranean environment. The genus *Allobathynella* is the most species-rich genus known in Korea, and it now contains 23 species and one subspecies from South Korea and Japan. In this paper, we described a new species of *Allobathynella* from Danyang, South Korea. *Allobathynella danyangensis* **sp. nov.** can be distinguished from its congeners by the presence of five simple setae on the antennule, seven spines on the maxillule and 3-5-10-6 setal formula of the maxilla. We describe the new species with molecular diagnosis based on the mitochondrial *c* oxidase subunit 1, the mitochondrial 16S rDNA, and the nuclear 18S rDNA gene sequences and morphological study.

Keywords

Crustacea, Korean peninsula, molecular data, stygofauna, taxonomy

Introduction

Parabathynellidae Noodt, 1965 belongs to the order of Bathynellacea, which is a common group of the stygofauna (Park and Cho 2013). They live in groundwater (caves, aquifers, wells, springs, and interstitial spaces between sand grains in riverbeds), on all continents except Antarctica and mechanisms and lack planktonic larvae (Camacho et al. 2012, 2014). These isolated habitats and the lack of active dispersal mechanisms have led to decreased dispersal distance, and hence, they consequentially show a high degree of endemism (Guzik 2008; Schminke 2014).

The genus *Allobathynella* has been considered as a primitive group of family Parabathynellidae in East Asia (Schminke 1973; Cho 2005; Park and Cho 2016). They have morphologically complex characters such as relatively large body lengths (1.28 mm–3.3 mm), multi-segmented antennule, antenna and thoracopodal exopod and wealth of appendicular ornamentation (Morimoto 1963; Fuchs et al. 2012; Park and Cho 2016). In particular, the multi-segmented exopod of thoracopods with more than two segments is a characteristic that occurs only in *Allobathynella* among the Korean parabathynellid genera.

The genus *Allobathynella* Morimoto & Miura, 1957 has been proposed for *A. japonica* from Japan (Morimoto 1957). It contains 23 species and one subspecies so far, including four species and one subspecies (*A. carinata* (Ueno, 1952), *A. kuma* (Uéno, 1956), *A. yaye* (Uéno, 1956), *A. gigantea* (Morimoto, 1959) and *A. gigantea pluto* (Morimoto, 1963)) incorporated into the genus *Parabathynella* Chappuis, 1926 (Uéno 1952, 1956, 1961; Morimoto 1959, 1963; Schminke 1973; Park and Cho 2008, 2016; Shin 2014). *Allobathynella* can be morphologically distinguished from *Parabathynella* by presence of one segmented thoracopod VIII in female and the presence of pleopod (Fuchs et al. 2012). All known species of *Allobathynella* are distributed in the Korean peninsula and Japan, and 17 species have been described in South Korea (Morimoto 1970; Park and Cho 2008, 2016; Shin 2014).

Based on the morphological examination of the specimens, here we report on a new species of *Allobathynella* found in the Hangang River in South Korea. In addition, we collected two related species, *A. hongcheonensis* Park & Cho, 2016 and *A. wonjuensis* Park & Cho, 2016, from type localities in the tributary of the Hangang River. We obtained mitochondrial cytochrome c oxidase subunit 1 (CO1), mitochondrial 16S rDNA and the nuclear 18S rDNA gene sequences from the new species and the two collected species and compared their morphological and molecular characteristics.

Materials and methods

Study area and groundwater sampling method

The samples were collected from the interstitial hyporheic zone of the Hangang River at three sites in South Korea: Danyang-gun, Hongcheon-gun, and Wonju-si, South Korea (Fig. 1B). The type locality of the new species, Danyang-gun, was a gravelly and rocky area rather than fine sandbanks (Fig. 1C, D). For sampling groundwater from the hyporheic zone, a 1 m core was driven into the points using a hammer, and water was collected using a manual pump. Approximately 80-100 L of the water sample was filtered through a 50 µm fine-mesh net. All the collected specimens were immediately preserved in 95% ethanol.



Figure 1. Map showing the type locality and habitat of the genus Allobathynella species and A. danyangensis sp. nov. A 1. A. imjinensis, 2. A. hongcheonensis, 3. A. bangokensis, 4. A. gangneungensis, 5. A. wonjuensis, 6. A. munmakensis, 7. A. buronensis, 8. A. donggangensis, 9. A. coreana, 10. A. yeonjuensis, 11. A. maseongensis, 12. A. yecheonensis, 13. A. munsui, 14. A. okcheonensis, 15. A. shinjongieei, 16. A. cheongdoensis, 17. A. gureeyensis, 18. A. carinata, 19. A. mirabilis, 20. A. japonica, 21. A. yaye, 22. A. gigantea pluto, 23. A. kuma, 24. A. gigantea gigantea B collection localities of the specimens used for the present study and the type locality of A. danyangensis sp. nov. C, D Collection sites of A. danyangensis sp. nov. at Danyang-gun, South Korea.

Morphological study

Specimens were dissected in glycerol under a stereo microscope (SZX12, Olympus, Japan). Dissected appendages were mounted using Eukitt Quick-hardening mounting medium (Sigma-Aldrich, St. Louis, MO, USA) for permanent slides. Observation and drawing were conducted using an optical microscope (DM2500, Leica, Germany). For scanning electron microscopy (SEM), the specimens were dehydrated in increasing concentrations of ethanol solutions, transferred into hexamethyldisilazane (Sigma-Aldrich, St. Louis, MO, USA), covered with platinum, and observed using a Hitachi S-4300SE (Hitachi, Japan). The type materials of the new species examined in this study were deposited in the collection at the National Institute of Biological Resources, Korea (NIBR).

Molecular analysis

The specimens used for the molecular study are listed in Table 1. Genomic DNA was extracted from the abdomens of specimens using the LaboPass Tissue Genomic DNA Isolation Kit Mini (Cosmo GENETECH, Seoul, South Korea) according to the manufacturer's instructions. Amplification by polymerase chain reaction (PCR) was conducted using the following primer sets: Bathy_F1 and Bathy_R1 for the CO1 mitochondrial gene (Ji et al. 2021); 16SarL F and 16SBathy-453R for the 16S mitochondrial gene (Palumbi et al. 1991; Perina et al. 2018); and two sets of 1F, 5R and 3F, 9R for the 18S nuclear gene (Giribet et al. 1996). These sequences were aligned using Clustal W (Thompson et al. 1994; Larkin et al. 2007) in Geneious v.8.1.9 (Biomatters, Auckland, New Zealand). The intra- and interspecific genetic distances were determined using MEGA X v.10.1.8 (Kumar et al. 2018).

Results

Taxonomy

Order Bathynellacea Chappuis, 1915 Family Parabathynellidae Noodt, 1965 Genus *Allobathynella* Morimoto & Miura, 1957

Allobathynella danyangensis sp. nov. https://zoobank.org/8146FEA6-E37B-4E4A-9535-307BD04992C5

Type locality. Danyang-gun (37°5'0.52"N, 128°28'57.11"E), South Korea. Collected by Su-Jung Ji, Chi-Woo Lee and Hee-Min Yang (19 June 2020 and 5 November 2021).

Type materials. *Holotype:* female (NIBRIV0000900570), dissected on six slides. Allotype: male (NIBRIV0000900577), dissected on five slides. *Paratypes:* Seven females (NIBRIV0000900571–3, NIBRIV0000900614–7) and five males (NIBRIV0000900574–6, NIBRIV0000900612–3).

Diagnosis. Antennule seven segmented with five simple setae on the inner distal margin of the third segment; antenna seven segmented with setal formula 0+0/0+0/1+0/1+1/0+1/1+1+1/5(1); labrum with 13 teeth; mandible palp one segmented with two apical setae; maxilla four segmented with a setal formula 3-5-10-6; thoracopods III–VII each with an epipod; uropod protopod with eight or nine spines and two distal spines slightly larger than other spines; furcal ramus with five spines; anal operculum slightly protruded.

Description of adult female (Figs 2–7). Body (Fig. 2) length 1.74 mm, head as long as three anterior thoracic segments combined.

Antennule (Fig. 3A) seven segmented, first segment with one small seta on inner distal margin, two simple dorsal setae of different sizes, and with four plumose setae on outer side; second segment with four simple setae on inner distal margin and one group of four plumose setae on outer margin; third segment with five simple setae on inner margin, with two simple lateral setae of different sizes and one lateral plumose seta; inner flagellum of third segment with three simple setae of different sizes; fourth segment with one stub seta and one plumose seta on dorsal margin and two plumose setae on the outer distal apophysis; fifth segment distally with four simple setae, two dorsal aesthetascs and one simple seta on inner margin; sixth segment with four setae on inner margin; sixth segment with four setae on inner margin, and with two aesthetascs, one simple seta, and one aesthetasc dorsally; and seventh segment with three subterminal aesthetascs and four simple setae.

Antenna(Fig. 3B) seven segmented; setal formula0+0/0+0/1+0/1+1/0+0/1+1+1/5(1).

Labrum (Fig. 3C) with eight median teeth flanked by two (left) or three (right) teeth on lateral sides; ventral surface with one small round median projection, three pairs of teats and numerous combs of ctenidia.

Mandible (Fig. 3D, E) with incisor process of four teeth; tooth of ventral edge absent; spine row consisting of eight spines; palp one segmented with two apical setae of different sizes, longer one being basally barbed; with one or two bundles of ctenidia that look like chestnut bur near the base of the palp.



Figure 2. Allobathynella danyangensis sp. nov., paratype female, NIBRIV0000900571. Scale bar: 0.5 mm.



Figure 3. *Allobathynella danyangensis* sp. nov., holotype female **A** antennule **B** antenna **C** labrum **D** mandible (dorsal, right one) **E** mandible (dorsal, left one). Scale bars: 0.5 mm.

Maxillule (Fig. 4A) two segmented, proximal segment with four setae on distal margin; distal segment with two terminal smooth spines; five dentated spines on inner edge, and three simple setae of different length on outer distal margin.



Figure 4. *Allobathynella danyangensis* sp. nov., holotype female **A** maxillule **B** maxilla **C** thoracopod I **D** thoracopod II **E** thoracopod III. Scale bars: 0.05 mm.

Maxilla (Fig. 4B) four segmented, setal formula 3-5-10-6.

Thoracopods I–VII (Figs 4C–E, 5) slightly increased in size up to thoracopod IV, thoracopods IV–VII similar in size; thoracopods III–VII each bearing one epipod on protopod; basipod with two distal setae in thoracopod I, with one distal seta in thoracopods II and III, and one distal and one median seta in thoracopods IV–VII; number of exopod segments of thoracopods I–VII: 3-4-5-6-6-6-6, with two setae on each segment, three in first segment of Th I; endopod of the thoracopods I–VII four-segmented, inner setae of first segment always plumose and all others smooth, setal formulae:

Thoracopod I	2 + 1/3 + 2/2 + 1/4(2)
Thoracopod II	2 + 1/3 + 2/0 + 1/4(2)
Thoracopods III–V	1 + 1/2 + 2/0 + 1/4(2)
Thoracopod VI	0 + 1/2 + 2/0 + 1/4(2)
Thoracopods VII	0 + 1/1 + 2/0 + 1/4(2)

Thoracopod VIII (Fig. 6A) conical in ventral view, with two sharp distal projections like teeth.

First pleopod (Fig. 6B) in form of stub bearing two distal plumose setae of different length.

Uropod (Figs 6C, 7D) bearing eight or nine spines on inner margin of sympod and two distal spines slightly larger than other spines; exopod 38% as long as the sympod length, with one outer seta, two terminal setae and one inner medial seta; inner setae strong, longer, and thicker than outer terminal seta; endopod longer than exopod, 52.8% as long as sympod with two dorsal plumose setae near base, two terminal setae and one subterminal plumose setae and with one terminal, and one subterminal spines and four additional spines.

Pleotelson (Fig. 6D) without seta; anal operculum slightly protruded.

Furcal rami (Fig. 6D) 1.3 times as long as wide, with two large distal spines and three smaller spines on inner margin, and with two dorsal plumose setae of different sizes.

Description of adult male (Fig. 7A–C). The male differs from the female in thoracopod VIII. Thoracopod VIII of male perpendicular to body, in the form of a bell in lateral view, 1.2 times longer than wide; protopod with a prominent penial region bearing distal opening; inner margin of penial region (dentate lobe) with five teeth (Fig. 7C, white arrow); epipod flat, with flat round distal part not reaching lower margin of the exopod; basipod with one seta near base of endopod, inner margin of basipod with distally drawn out into one projection, and basipodal seta as long as endopod; exopod one- third of basipod, round, with two distal lobes; the two lobes with tiny denticles (Fig. 7C, yellow arrow); endopod small, round, with two distal setae of different sizes.

Remarks. Allobathynella danyangensis sp. nov. is morphologically most similar to *A. coreana sensu* Park and Cho (2016) as follows: 1) the antennule third segment has two simple setae and one plumose seta on the outer distal margin, 2) the last segment of the antenna has five setae, 3) mandibular palp is one segmented, and 4) male thora-



Figure 5. *Allobathynella danyangensis* sp. nov., holotype female **A** thoracopod IV **B** thoracopod V **C** thoracopod VI **D** thoracopod VII. Scale bars: 0.05 mm.

copod VIII has one long basipodal seta. However, the new species can be differentiated from *A. coreana* by the following characteristics (characters of *A. coreana* in parentheses): 1) the antennule third segment has five (four) simple setae, 2) the mandibular palp has two (one) apical setae, 3) the maxillule has seven (eight) spines on the distal segment and 4) the third segment of the maxilla has 10 (12) setae.



Figure 6. *Allobathynella danyangensis* sp. nov., holotype female **A** thoracopod VIII **B** pleopod **C** uropod **D** telson. Scale bars: 0.05 mm.

The new species is morphologically also closely resemble *A. hongcheonensis* Park & Cho, 2016 as follows: 1) the antennule third segment has two simple setae and one plumose seta on the outer distal margin, 2) the mandibular palp is one segmented and has two apical setae and 3) maxillule has seven spines on the distal segment. However,



Figure 7. *Allobathynella danyangensis* sp. nov. (**A**) paratype male, NIBRIV0000900574, (**B**) paratype male, NIBRIV0000900575, (**C**) paratype male, NIBRIV0000900576, (**D**) paratype female, NIBRIV0000900617 **A** thoracopod VIII (ventral view) **B** thoracopod VIII (lateral view) **C** Thoracopod VIII (ventral view) **D** uropod. Scale bars: 0.05 mm (**A**); 0.02 mm (**B**, **C**); 0.1 mm (**D**).

the new species differs from *A. hongcheonensis* in the following characteristics (characters of *A. hongcheonensis* in parentheses): 1) the second segment of maxilla has five (four) setae, 2) thoracopod VIII of female has two sharp distal projections (two distal lobes with denticles) and 3) thoracopod VIII of male has one long (tiny) basipodal seta.

Etymology. The species name is derived from Danyang-gun, where the material was collected.

Allobathynella hongcheonensis Park & Cho, 2016

Material examined. Collected in the type locality (37°41'57.5"N, 127°40'10.4"E) by Chi-Woo Lee (25 March 2015). One female specimen was examined (NIBRIV0000900580). Although the specimen differs from the original description of the species in having eight spines on the mandible spine row instead of nine, five spines on the endopod of the uropod instead of six, and five spines on the furcal ramus instead of six, it is within the range of intraspecific variability. In addition, the present specimen morphologically differs from *A. bangokensis* Park & Cho, 2016, which is a sympatric species with *A. hongcheonensis*, in the antenna, maxillule and maxilla. Thus, we identified the studied specimen as *A. hongcheonensis*.

Allobathynella wonjuensis Park & Cho, 2016

Material examined. Collected in the type locality (37°22'34.1"N, 127°51'15.2"E) by Chi-Woo Lee (25 March 2015). Two female specimens were examined (NI-BRIV0000900578–9). The two specimens are consistent with the original description of the species, except having nine spines on the uropod sympod instead of eight in NIBRIV0000900578. Therefore, we identified the studied specimens as *A. wonjuensis*.

Molecular analysis. We sequenced and analyzed DNA extracted from the new species and the two collected species (Table 1). A total of 786 bp for the mitochondrial CO1, 452 bp for 16S rDNA and 1704 bp for the 18S rDNA gene. The uncorrected pairwise distances within and among the species of the genus *Allobathynella* are shown in Table 2. In the analyzed species, the ranges of interspecific variation for CO1, 16S and 18S were 16.8–19.8%, 19.1–21.7% and 0.2%, respectively (Table 2).

Discussion

The species of the genus *Allobathynella* are distributed across South Korea and Japan and occurred mostly in interstitial groundwater habitats at the riverbanks in South Korea, and in spring or driven well habitats in Japan (Fig. 1A). Seven *Allobathynella* species were found distributed in the northwestern part of South Korea along the course of the Hangang River, a major Korean river (Fig. 1B). These species were *A. bangokensis*, *A. hongcheonensis*, *A. wonjuensis*, *A. munmakensis*, *A. buronensis*, *A. coreana*, and

Species, sex	Locality (Coordinates)	Date	Voucher No.	GenB	ank accessio	on No.
				COI	165	185
<i>A. danyangensis</i> sp. nov., holotype female	Danyang-gun, South Korea (37°5'0.52"N, 128°28'57.11"E)	2021.11.05	NIBRIV0000900570	OP214600	OP214779	OP214784
<i>A. danyangensis</i> sp. nov., paratype female	T	2021.11.05	NIBRIV0000900571	OP214601	OP214780	OP214785
<i>A. danyangensis</i> sp. nov., paratype female (juvenile)	п	2020.06.19	NIBRIV0000900572	OP214602	-	-
A. hongcheonensis, female	Hongcheon-gun, South Korea (37°41'57.5"N, 127°40'10.4"E)	2015.03.25	NIBRIV0000900580	OP214603	OP214781	OP214786
A. wonjuensis, female	Wonju-si, South Korea (37°22'34.1"N, 127°51'15.2"E)	2015.03.25	NIBRIV0000900578	OP214604	OP214782	OP214787
A. wonjuensis, female	"		NIBRIV0000900579	OP214605	OP214783	OP214788

Table 1. Samples used for the molecular analyses, with collection locality and date, voucher numbers and GenBank accession numbers.

Table 2. Intra- and interspecific genetic distances of three molecular markers (CO1, 16S rDNA and 18S rDNA) (*p*-distance) among the new species and two *Allobathynella* species obtained in the present study.

C01	Intraspecific (%)		Interspecific (%)	
Species name		1	2	3
A. danyangensis sp. nov.	0-0.5	-		
A. hongcheonensis	-	16.8-16.9	-	
A. wonjuensis	1	19.0-19.5	19.5-19.8	_
165	Intraspecific (%)		Interspecific (%)	
Species name		1	2	3
A. danyangensis sp. nov.	0	-		
A. hongcheonensis	-	21.7	-	
A. wonjuensis	0	21.6	19.1	_
185	Intraspecific (%)		Interspecific (%)	
Species name		1	2	3
A. danyangensis sp. nov.	0	-		
A. hongcheonensis	-	0.2	-	
A. wonjuensis	0	0.2	0.2	-

A. donggangensis (Morimoto 1970; Park and Cho 2016) and may be closely related to each other. Comparison of the morphological features of the eight *Allobathynella* species, including the two species collected in the present study and the new species, is provided in Table 3. On the other hand, *A. coreana* has been detected at four sites in South Korea to date: Yongdam-gul Cave, Kwangcheon-seon-gul Cave, driven well at Ka'eun-myeon and the hyporheic zone of Yeongwol-gun, South Korea (Morimoto 1970; Park and Cho 2016). Although the four distribution sites of *A. coreana* are geographically close to the type locality of the new species, the four forms have been described in the original paper without sufficient morphological details of important taxonomic characters, and the new species is morphologically distinct from all four forms of *A. coreana* (Table 3). Furthermore, considering their morphological differences based on available data, and that the populations from each locality are geographically isolated and may not interact with each other, the four forms of *A. coreana* may be separate species. Therefore, taxonomic re-examination and molecular data are required to estimate the true species diversity, with possible existence of cryptic species, and understand their distribution ranges.

Characteristics of A. coreana include the	
hynella from a tributary of Hangang River, South Korea. (
ong eight species of Allobat	
. Morphological differences ame	yraphical forms.
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rour geogr	raphical forn	ns. <u>A hanaohoneis</u>	4 humaneie	V	V	V	4 moninencie		4 00	04004		4 damanancie
		TI UNING WEININ	C101010100 117	donggangensis	hongcheonensis	mumakensis	-	Cave	Cave	Ka'eun-myeon	Yeongwol-gun	sp. nov.
								Yongdam	Kwangcheon		1	
Antennule	No. of	7	~	7	7	7	6	~	7	7	~	7
	segment											
	Inner margin of 3 rd	3 simple setae	5 simple setae	3 simple setae	4 simple setae	4 simple setae	4 simple setae	~ •	۰.	۰.	4 simple setae	5 simple setae
	segment											
	Outer margin	2 simple setae,	2 simple setae,	1 simple setae,	2 simple setae,	2 simple setae,	2 simple setae,	~·	~·	~·	2 simple setae,	2 simple setae, 1
	of 3rd segment	t 1 plumose seta	1 plumose seta	1 plumose seta	1 plumose seta	1 plumose seta	1 plumose seta				 plumose seta 	plumose seta
Antenna	Setal formula	1 0-0-1-2-0-3-5	0-0-1-2-0-3-5	0-0-1-2-1-2-4	0-0-1-2-0-3-5	0-0-1-2-0-3-5	0-0-1-2-0-3-4	0-0-0-0-1-1-4	0-0-1-2-1-2-4	0-0-0-1-1-3-5	0-0-1-2-0-3-5	0-0-1-2-0-3-5
	Ctenidia in	present	absent	absent	absent	absent	absent	<u>~</u> .	۸.	λ.	absent	absent
		Ì		ļ		Ì	Ì	:	c			
Labrum	No. of teeth	14	10	17	13	14	14	11	6	10	14	13
Mandible	Palp segment	- 1	1	1	1	1	1	1	1	1	1	1
	No. of seta	2	2	1	2	1	1	2	2	2	1	2
Maxillule	No. of spines on distal	∞	4	9	7	~	7	9	7	7	ø	7
	segment											
Maxilla	Setal formula	1 3-5-11-6	3-4-10-6	3-5-9-6	3-4-10-6	3-5-11-6	3-4-9-6	3-3-7-6	3-4-9-6	3-4-8-7	3-5-12-6	3-5-10-6
Thoracopod	Epipod in	IIV-VI	IIV-III	IIV-VI	III-VII	III-VII	III-VII	IIV-III	III-VII	IIV-VI	IIV-III	IIV-III
Uropod	No. of spines	10	6	10	7	6	8	10	8	12	12	8–9
	on sympod											
Furcal	No. of spines	9	5	6-7	5-6	9	5	5	9	5	9	5-6
ramus												
Ref.		Park & Cho, 2016	Park & Cho, 2016	Park & Cho, 2016	Park & Cho, 2016; this	Park & Cho, 2016	Park & Cho, 2016, this	Morimoto, 1970	Morimoto, 1970	Morimoto, 1970	Park & Cho, 2016	This study
					study		suuuy					

In our specimens, the young individuals resembled adults and had thoracopod VIII acting as a reproductive organ. However, they still lacked segments on the exopods of the thoracopods. All the specimens that we considered as adults and described had the 3-4-5-6-6-6-6 exopod segment formula, but the juveniles had formulae of 3-4-4-5-5-5-4, 3-4-5-5-5-4 and 2-3-4-5-5-4-3. Observation of morphological traits and analysis of gene sequence data from a juvenile indicate that they are the same species as the present new species (Tables 1, 2). They seem to acquire the rest segments of the exopods of thoracopods through subsequent moulting (Schminke 1974). Progenesis is defined as the sexual maturation of organisms at a morphologically juvenile or larval stage (Gould 1977). In general, progenesis is regarded as an important role in the evolution of interstitial organisms (Schminke 1973; Westheide 1987). Pressure for small size in the interstitial space is estimated as the primary factor (Gould 1977), and this is also considered to be the most convincing opinion for the regressive morphological status of interstitial taxa (Westheide 1987). Thus, the segment formula of thoracopod exopods can be a taxonomically inadequate character when judging them as adults as their genital organs are mature or when describing them without examining enough specimens.

The previous classification of the order Bathynellacea from Korea has been investigated using only a morphological approach while the recent studies suggest combining both morphological and genetic analysis to characterize genera and species or reveal their phylogenetic relationships (Camacho et al. 2013; González-Miguéns et al. 2020). To examine the genetic divergence within and between the present new species and the two collected species, we sequenced 786 bp of the mitochondrial CO1, 452 bp of 16S and 1704 bp of the 18S gene (Tables 1, 2). The small distance found in the 18S gene tells us that the three species definitely belong to the same genus and the distances of the COI and 16S mitochondrial genes, undoubtedly show us that they are three different species. It is necessary to sequence genes from the type localities of nominal species, well characterized morphologically, in order to make adequate comparisons with morphologically similar species from other localities. Only in this way, combining molecular and morphological data, will it be possible to understand the true diversity of the group. Our result provides a basis for future comparison with other Bathynellacea species and contributes to phylogenetic studies.

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RESEARCH ARTICLE



First biological data, associated fauna, and microclimate preferences of the enigmatic cavedwelling beetle *Dalyat mirabilis* Mateu, 2002 (Coleoptera, Carabidae)

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Abstract

Dalyat mirabilis is an extraordinary troglobite carabid described in 2002 from the cave Simarrón II in the southeast of the Iberian Peninsula (Spain). A new subfamily Dalyatinae was erected to accommodate this species with remarkable morphological characters and adaptations to live underground. In addition to the former original descriptions, there is only one more study and it aimed to elucidate its evolutionary history. Its closest living relative belongs to the genus Promecognathus in North America and both groups seem to have diverged sometime in the late Jurassic to early Cretaceous. In this work, the phenology of D. mirabilis, its associated invertebrate fauna and the environmental conditions of the cave Simarrón II were studied for a full year cycle. This carabid is not evenly distributed in the cave, in time or space. It is most abundant during the winter months, wet season, and it disappears from the top layer of the substrate in the summer. A positive correlation was found between the number of carabids captured per trap and the distance to the entrance of the cave; most specimens were captured in traps farthest from the entrance and located in the chamber known as Vias Salas Negras. Furthermore, several spatially-resolved analyses integrating relative humidity, temperature, and the number of captures per trap showed that D. mirabilis prefers Vias Salas Negras for having a higher and more stable relative humidity than other chambers in the cave. Larvae were never captured, regardless of intense efforts to collect them for years. Finally, 30 other invertebrate species belonging to 12 different Orders were captured in the cave and are listed here, 25.8%

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are troglobites, 29.0% troglophiles and 45.2% troglexenes. The data from this study was used for an initiative to protect this cave and its remarkable fauna. Some of the measures taken by the Administration include the control of human visits to the cave, the installation of a perimetral fence surrounding the entrance, and the installation of an informative panel at the exterior of the cave describing the endemic entomological fauna it contains.

Keywords

Sierra de Gádor, Simarrón II, Spain, troglobite

Introduction

Dalyat mirabilis Mateu (Coleoptera: Carabidae) (Fig. 1) was discovered in the year 2000 in a series of biological explorations of caves in the south-eastern of Spain, in the province of Almería. It was described as a new species in 2002 based on a handful of specimens and a new subfamily Dalyatinae was also erected to accommodate this singular species (Mateu 2002). According to its morphological features, D. mirabilis relates particularly to five other members in the subfamily Promecognathinae, one genus is present in North America and four genera in South Africa (Mateu and Bellés 2003). The adaptations to live underground, like the complete absence of eyes and the elongation of appendages (legs and antennae), clearly separates this beetle from its closest relatives in Promecognathinae. A molecular phylogenetic study using 18S rRNA and a fragment of *wingless* concluded that there was a strong support for a sister relationship between the newly erected genus *Dalyat* and *Promecognathus* (genus present in North America). Using a molecular-clock approach the two lineages were estimated to have diverged at a similar age, or slightly earlier than the origin of the subfamily Harpalinae, known to have radiated in the Cretaceous. This is compatible with a vicariant origin of the lineage leading to Dalyat because of the isolation of the Iberian plate from Pangea in the late Jurassic to early Cretaceous (Ribera et al. 2005).

D. mirabilis is a true troglobite beetle known from only four locations: Cueva Simarrón II, Cueva de los Chupones, Cueva del Cementerio and an old water mine Fuente Vieja; all of them in Sierra de Gádor (southeast of the Iberian Peninsula). The farthest distance in a straight line between the four locations is no more than 8 kilometers, however these caves are at a very different altitude, with more than 1,000 meters difference in altitude. It has only seen alive and in relatively high numbers in the cave Simarrón-II (Mateu and Bellés 2003). The habitat of *D. mirabilis* and its morphological features, point this species as a strict cavernicole (Mateu and Bellés 2003).

There are only three studies performed on *D. mirabilis*, two morphological descriptions and the molecular analysis that elucidated its phylogenetic origin (referenced above). Unfortunately, nothing is known about the biology, distribution in the cave, environmental requirements, feeding habits, or the population size of this enigmatic species. From a conservation point of view, this information is relevant to take further protective measures in the future.



Figure 1. Adult specimen of Dalyat mirabilis (Mateu 2002) in the cave Simarron II.

In the present study, data about the phenology of the species, distribution in the cave Simarrón II, an explanation for the distribution pattern observed, and other arthropods associated with *D. mirabilis* in the cave are described. The new information contributes to the knowledge of *D. mirabilis* in the only cave the species has been found alive in high numbers and it will help prioritize future conservations efforts to preserve this unique species.

Materials and methods

The phenology of *D. mirabilis* and its associated invertebrate fauna was studied in the cavern Simarrón II for a full year cycle, from February to January. Simarrón II is located in the municipality of Dalías, Almería (Spain) at 1,480 m.a.s.l. and in a south-facing slope of Sierra de Gádor. The explored portion of the cave spans for about 453 m (Fig. 2).

Simarrón II was sampled twice per month using dry baited pitfall traps set in the first visit and collected two weeks after. Pitfall traps consisted of a tapered plastic container (8.5 cm diameter at the top) inserted into a cylindrical container of the same diameter. The whole pitfall trap was buried in the soil up to its upper rim and baited with sobras-sada (Fig. 3). Sobrassada is a smelly spicy sausage mainly composed of pork fat with some



Figure 2. Location of the cave Simarrón-II in Spain, trap numbers and placement locations (numbers in circules) in the cave, as well as temperature and relative humidity measuring stations (letters).

preservatives to avoid fungus and bacterial colonization. It has been shown to be a long lasting and attractive bait for insects (Ortuño and Barranco 2015; Jordana et al. 2017).

Beetles captured alive (Fig. 4) were marked with a unique code using Tipp-ex[®] and released in the proximity of the trap they were captured. Tipp-ex[®] was used because is easy to use to mark specimens and dries up in only a few seconds (2–5 seconds). Small rocks were placed at the bottom of the collection chamber of the trap to serve as a refugee to avoid predation among specimens captured. Baited traps were distributed along the cave with an emphasis on the chambers "Salas Vias Blancas" and "Salas Vias Negras" (Fig. 1). Air temperature and humidity were recorded with a HANNA Instruments HI 8564 thermo hygrometer (resolution 0.1 °C and 0.1%, precision range ± 0.4 °C and $\pm 2\%$ RH) in every visit to the cave and at each station with a pitfall trap (Fig. 1). A survey for invertebrates, other than *D. mirabilis*, was conducted in every visit to the cave by direct examination of the substrate, loose rocks, gours, guano, and places with accumulation of organic matter. Around 20–60 minutes were spent sampling the arthropods in the surroundings of each pit fall trap station. Collection of specimens was limited to those necessary for identification.

To explore the distribution of *D. mirabilis*, a single sampling trip to determine the presence/absence of the species during the wet season (highest abundance of the

species) was carried out in the caves: Cueva del Cementerio and Cueva de los Chupones (Municipality of Berja), Fuente Vieja (Municipality of Dalías) and Cueva del Águila (Municipality of Berja). All of them are located relatively close to Simarrón II.

The beetle's habitat was characterized in terms of the prevailing temperature and relative humidity of the cave atmosphere Simarrón II. Firstly, the number of specimens of *Dalyat mirabilis* were gridded and interpolated using the inverse distance algorithm. The spatial distribution was represented with a XY Contour Data map using GrapherTM (Golden Software, LLC). The XY coordinates correspond to the cave air temperature and relative humidity, respectively. Secondly, box and bubble plots were used to assess the absence or presence of specimens and its relative abundance in function of the cave microclimate stability.



В

Α



Figure 3. Schematic representation of pitfall traps used (**A**) and pitfall traps located in Salas Negras (**B**) during this study to capture live specimens of *D. mirabilis*. Note the rocks and substrate added inside the trap to avoid predation among specimens captured.



Figure 4. Content of one of the pitfall traps after 15 days in the cave Simarron II and containing 6 specimens of *D. mirabilis* (one under the rocks).

Results and discussion

Environmental conditions of Simarrón II

Temperature in Simarrón II was quite stable and the annual averages obtained were 11.7, 11.7, 11.6, 12.0 and 11.9 °C for the sampled stations A, B, C, E and F, respectively (Fig. 5A). Stations A, B, and C correspond to the chamber Via Salas Blancas, and temperatures were slightly colder (~0.3–0.4 °C) than those measured in the stations E and F of Via Salas Negras. The annual fluctuations were 0.13 and 0.19 °C for stations A and B, 0.43 °C for station C, and 0.21 °C for both stations, E and F. The high fluctuations observed in the station C maybe explained by the presence of small cracks at the top of this chamber that connects to the exterior of the cave, as evidenced by organic matter and plant debris found in this area.

The average annual relative humidity (RH) recorded for stations A, B, C, E and F were 81.3%, 82.9%, 86.6%, 90.5% and 91.2% respectively (Fig. 5B). Overall, Via Salas Blancas showed a slightly lower RH (ave = 83.6%) than Via Salas Negras (ave = 90.8%). The annual fluctuations calculated were 9.8%, 10.6% and 7.8% for the sampling stations (A, B, C) in Via Salas Blancas, and 2.0% for stations (E, F) in Via Salas Negras.


Figure 5. Temperature (**A**) and relative humidity (**B**) data measured at the stations (**A–F**) for a full cycle in the cave Simarrón II.

RH went down to 64.8% during the summer in Via Salas Blancas (station A), while the lowest RH recorded in Vias Salas Negras was 87.4% (station E). The differences between the two areas of the cave are remarkable, and could potentially determine the arthropod-fauna present in each of them. Consistent with a cave located in the Mediterranean basin, humidity decreased in the months of the summer (July-October, dry season) and increased to peak in the months of February-March when the rain and melted snow arrived. In general, during the fall (beginning of the next rainy season of next cycle), the relative humidity increased again to similar levels recorded in previous winter.

Dalyat mirabilis in the cave Simarrón II

A total of 119 specimens of *D. mirabilis* were collected in this study (without counting the recaptures): 89 were captured in pitfall traps and 30 were direct captures. Fourteen specimens were death or predated by other specimens inside the traps. From the carabids captured alive, 53 were marked by a unique code, five were released without taking any action, and 25 were brought to the laboratory for further biological studies. These were sent to the "Departamento de Ciencias de la Vida" in the "Universidad de Alcala de Henares", Madrid (Spain) for feeding, ethological and biological studies. Despite that many of the specimens survived under lab conditions for several months, no mating was observed, and no eggs or larvae could be obtained. Very aggressive behavior was detected among specimens of the same and also opposite sex. Interestingly, D. mirabilis uses their mandibles to transport small stones to build a shelter where they remain most of the time. We never observed D. mirabilis feeding in its native habitat (cave Simarrón II), but it was previously reported that North American ground beetles of the genus Promecognathus prey on polydesmid millipedes (Weary and Will 2020). D. mirabilis is a troglobite inhabiting caves exclusively, and millipedes have not been captured in any of the four caves/locations where D. mirabilis has been reported. The most abundant and possible prey of *D. mirabilis* in the cave Simarrón-II is the isopod (Trichoniscus sp.). Specimens of D. mirabilis kept in the laboratory were fed successfully dipteran larvae and the aforementioned isopod.

From the 53 carabids that were marked, only four were recaptured in this study.

The number of specimens of *D. mirabilis* observed at different months and stations sampled in the cave fluctuated widely. The highest number of captures were recorded during the winter months. The number of captures dramatically decreased during the summer months, reaching a minimum in September (only month without captures) (Fig. 6A). The most effective trap was pitfall number 8 (32 captures) and followed by 7 (20 captures), both located in Via Salas Negras. Pitfalls 5 and 9 followed with less than 50% of the captures of pitfall 8 (Fig. 6B). Traps 1 (1 capture) and 2 (no captures) had the least amount of captures, both located in Via Salas Blancas (Fig. 6B).

We explored the possible relationship of the distance of each pitfall trap location to the entrance of the cave and the number of carabid captures obtained per trap (Fig. 7). A direct and positive correlation was found, the highest number of captures were in pitfalls farther away from the entrance (stations F, E and C) (Fig. 7). Pitfall traps 1, 2 and 3 located in stations A and B were the closest to the entrance of the cave and also with the least amount of beetle captures (Fig. 7).

In general, an estimation of the population size of soil arthropods based on using pit-fall traps alone is difficult because it is affected by several factors, including how motile the species subject of the study is. However, several pit-fall traps placed



Figure 6. Total number of captures of *Dalyat mirabilis* per month (**A**) and per pitfall trap (**B**) during the period of the study the cave Simarrón II.

strategically on a grid could be used to get an approximation to it (Raworth and Choi 2001). Mathematical models could be used to estimate the population size using properly spaced pit-fall traps distributed using a spatial system in crossed axes (Perner and



Figure 7. Box-plot representing the abundance and variability of *Dalyat mirabilis* in function of the distance to the cave entrance along each sampling station.

Schueler 2004). The main problem for the design of such a system in a small/medium sized cave is that the possible location of the traps is limited by the development of the cave and the human ability to progress through it. Tercafs and Brouwir (1991) used this method successfully to estimate the population of other cave-dwelling beetles.

Simarrón II has narrow, tortuous and complicated paths, and it is made of large blocks and little or no substrate which makes the placement of traps extremely difficult. Perhaps, this has determined the low number of recaptures in this study, only four specimens in the entire sample period. The low number of beetles recaptured invalidate the application of any formula/models used previously to determine population size, and even if applied, the results would not be relevant using such a low recaptured numbers. It was not optimal that several individuals (14) were found dead in the traps, some live specimens were taken for laboratory experiments (25) and that not all individuals captured alive were marked (5). An observation we made of this species is that it is extremely territorial and aggressive. This may explain the presence of so many dead individuals in the traps (usually body parts); it is a very confined space for several territorial individuals trapped. There is an important presence of this species in Salas Negras, this is justified by the relatively high number of specimens captured in this part of the cave. However, as mentioned previously, the number of recaptures is low. Other than the explanations given above for this low number, it is also possible that the population size is relatively big, and therefore new "non-marked" specimens are always captured in each sampling trip. This will also imply that the specimens do not "hang around" the area where the trap is located since they are not recaptured, and instead, they keep moving within the interstice of the cave (they are not seeing again). It is an unlikely possibility that marked and released specimens learn to do not fall again in the traps. In any case, future studies will address some problems encountered here and will aim to determine the population size of this enigmatic species.

Environmental conditions of Simarrón II and distribution of D. mirabilis

Several spatially-resolved analyses were performed to understand the distribution of *D. mirabilis* in the cave Simarrón II. A distribution map integrating relative humidity, temperature, and the number of captures per trap was created (Fig. 8). This map indicate that the most abundant captures happened in areas of the cave with high relative humidity (>82%), and air temperatures between 11.25 °C and 12.25 °C (Fig. 8). This may indicate that *D. mirabilis* prefers the inner, and most climatically stable areas of the cave, in agreement with the general trend that species richness and diversity of troglobionts are typically greatest deeper inside caves (Kozel et al. 2019).

To further understand the influence of each variable, we analyzed independently whether strong fluctuations in the temperature or relative humidity determine the presence/absence or abundance of *D. mirabilis*. In a first analysis, a strong positive correlation was found for pitfalls with a high number of *D. mirabilis* captured and environmental conditions with a low coefficient of variation (CV) of the relative humidity at those sampling stations. Those stations with the highest number of captures were in areas of the cave with the lowest fluctuations of relative humidity (Fig. 9). We could not find a clear correlation when a second analysis was carried out using the same captures per station and the CV (%) of the cave air temperature. Stations with the highest and also lowest number of captures had relatively stable temperature; however, station C that has the highest annual variation in the air temperature recorded more specimens than station A and B with lowest number of captures but more stable temperature (Fig. 10).

Pitfall traps located in Salas Negras away from the entrance showed more stable environmental conditions: higher and more stable relative humidity compared to Salas Blancas, higher number of captures (% of the total) and a slightly higher and stable temperature (Figs 5, 8, 9 and 10). In fact, this is the only area of the cave that the substrate and walls does not dry out in the summer months; and the environmental conditions (humidity and temperature) favored the presence of *D. mirabilis* all year round. These results suggest that the presence of *D. mirabilis* maybe determined mostly by a high and relatively stable relative humidity. This



Figure 8. Distribution map for the number of specimens of *Dalyat mirabilis* depending on the cave air temperature and relative humidity. Estimations of the number of specimens are based on the sampling (captures) and temperature and relative humidity records at each station (labelled as **A**, **B**, **C**, **E** and **F**) during an annual cycle, and using the inverse to a distance gridding calculation.

is in line with some early studies on the distribution of cave-inhabiting terrestrial arthropods. It was reported that most abundant populations are usually a short distance inside the dark zone of a cave entrance (Peck et al. 1976). This is where certain microclimatic stability is reached, i.e. contrary to what might be expected, which would be to find a greater species diversity and larger population sizes in the most external parts of the cave with the greatest food availability. A more stable cave environment also favors some species over others; especially species with high specialization for cave life, or edaphic spaces (Tobin et al. 2013; Pellegrini et al. 2016). Other studies suggest that the distribution of several epigeous species (trogloxenes or accidentals) is also linked to cave features (e.g. cave morphology and humidity conditions, among others), and follows patterns that are similar to the ones observed for more usual cave dwellers (Lunghi et al. 2014). Since the temperature was more or less stable across the cave (Figs 2, 5), more studies with a broader range of temperatures are needed to determine the preference of *D. mirabilis*, and its tolerance to seasonal fluctuations.



Figure 9. Bubble plot of the presence and abundance of *Dalyat mirabilis* as a function of the coefficient of variation (CV, %) for the relative humidity. The coefficient of variation represents the percentage of the standard deviation to the mean, and it is a useful statistic for comparing the degree of microclimatic stability (in terms of time-variation of humidity) from one location to another into the cave. The size of the blue circles indicates the relative abundance of *Dalyat mirabilis*. The dashed linear function (slope: -0.87, y-axis intercept: 10.20 and R² = 0.81) shows a remarkable increment of presence and abundance of *Dalyat mirabilis* as the CV of relative humidity decreases below 3%.

Associated fauna to D. mirabilis in the cave Simarrón II

The invertebrate fauna that Simarrón II hosts is unique and with a high level of endemism. A total of 31 invertebrate species were identified in this cave and two are in the process of being described as new species (Table 1). The 31 species includes 8 (25.8%) obligate cave dwellers (troglobites), 9 (29.0%) facultative cave dwellers (troglophiles) and 14 (45.2%) are animals found in caves, but also on the surface, where they encounter proper conditions for their life cycles (trogloxenes).

In addition to *D. mirabilis*, there is another coleopteran of the family Carabidae, *Platyderus speleus*, captured in Simarrón II. This species was originally described from the touristic Caves of Nerja in south Spain (Malaga). It has also been captured in three other caves in the province of Almería (data not published). This is an emblematic species since it is one of two species present in Spain of the genus *Platyderus* that shows a



Figure 10. Bubble plot of the presence and abundance of *Dalyat mirabilis* as a function of the coefficient of variation (CV, %) for the cave air temperature. The coefficient of variation represents the percentage of the standard deviation to the mean, and it is a useful statistic for comparing the degree of microclimatic stability (in terms of time-variation of temperature) from one location to another into the cave. The size of the blue circles indicates the relative abundance of *Dalyat mirabilis*.

high adaptation to live in caves (Zaballos and Jeanne 1994), although it can be found outside caves under big rocks too (Anichtchenko 2009).

Potential preys of these two carabids are 8 species of collembolans captured in Simarrón II (Martínez et al. 2004). They are edaphic and troglophiles and distributed along the cave. Some of the species, *Arrhopalites pygmaeus* or *Gisinurus malatestae* are commonly found in the soil in Europe or in Mediterranean countries. *Yoshiiphorura bellingeri* is distinct from other species known and represents a new genera and species described from specimens captured in three caves located at high altitude in Sierra de Gádor (all of them over 1,600 m.a.s.l.). It is a troglophile species, not associated with guano, and it was captured in pitfall traps during all year; however, the number of specimens captured in summer was considerably lower than in the other months.

Two singular species of pseudoscorpions, *Chthonius mayorali* and *Neobisium piquerae*, were captured in Salas Blancas. *Ch. mayorali* was associated with dryer areas in this big chamber, while *N. piquerae* occupied soil and substrate that is permanently wet. In the winter, *Ch. mayorali* was only found in the upper part of the chamber Salas

Phylum	Class/Order	Species	Occurrence
ARTHROPODA			
	Insecta		
	Order Coleoptera	Dalyat mirabilis	troglobite
		Platyderus speleus	troglobite
		Laemostenus baeticus	trogloxene
		Otiorhynchus sp. nov.	troglobite
	Order Diplura	Plusiocampa gadorensis	troglobite
	Order Thysanura	under study	trogloxene
	Order Collembola	Yoshiiphorura bellingeri	troglophile
		Mesogastrura ojcoviensis	troglophile
		Acherontiella xenylliformis	troglophile
		Arrhopalites pygmaeus	troglophile
		Onchopodura crassicornis	troglophile
		Gisinurus malatestae	troglophile
		Troglopedetes machadoi	troglophile
		Orchesella cincta	troglophile
	Order Psocoptera	Psillococus ramburi	trogloxene
	Order Orthoptera	Petaloptila barrancoi	trogophile
	Order Diptera	Heteromyza atricornis	trogloxene
		Dixella attica	trogloxene
		Trichocera maculipennis	trogloxene
	Order Siphonaptera	Leptopsylla taschenbergi amitina	trogloxene
	Arachnida		
	Order Pseudoscopionida	Chthonius mayorali	troglobite
		Neobisium piquerae	Troglobite
	Order Araneae	Tegenaria herculea	troblobite
	Order Acari	Androlaelaps fahrenholzi	trogloxene
		Eulaelaps novus	trogloxene
		Uroseius acuminatus Dn	trogloxene
		Haemogamasus nidi	trogloxene
		Galeolaelaps helianti	trogloxene
		Proctolaelaps pygmaeus	trogloxene
	Malacostraca		
	Order Isopoda	Porcellio sp.	trogloxene
		Trichoniscus sp. nov.	troglobite

Table 1. List of arthropod species captured and identified in the cave Simarrón II.

Blancas flowstone, probably displaced by *N. piquerae*, that is normally spotted and captured in the lower portion of this space. They are both endemic of Sierra de Gádor and *C. mayorali* has being captured in two other caves, and *N. piquerae* has been reported only from one more cave (Cueva de la Corraliza) where both species also coexist.

The isopod *Trichoniscus sp.* is very abundant in the deepest chamber of Salas Negras all year round, especially in a pile of fresh bat guano where traps 9 and 10 are located. *Trichoniscus sp.* was captured in traps but also by hand capture. Predation on this species by *D. mirabilis* has been observed in the laboratory. This isopod will be described as a new species.

The only identified spider, *Tegenaria aff. herculea* shows troglomorphic adaptations, such as eye size reduction, elongation of appendages and depigmentation (Ribera et al. 2000). There are two other endemic species captured in Simarrón II that are widespread in other caves in Sierra de Gádor. The first one is the cricket *Petaloptila barrancoi* that has been captured in more than twenty caves in these mountains (Barranco and Amate 2008). It is a troglophile species (Barranco and Molina-Pardo 2021) that does not usually go deep in the cave, and it was only found in the chamber Salas Blancas in Simarrón II. The second species is the dipluran *Plusiocampa gadorensis*, a troglobite species captured along the entire cave of Simarrón II (Sendra et al. 2020). It has also been captured in 10 more caves in Sierra de Gádor: Cueva de la Mudica, Sima Termal, Cueva and Sima del Llano de la Montés, Cueva de la Corraliza, Cueva Nueva, Sima del Aire, Sima del Puntal, Cueva de las Colmenas-II and the artificial water mine Fuente Vieja (data not published).

The flea *L. taschenbergi amitina* is a common parasite of species of rodents in the genus *Apodemus* (Gómez et al. 2013); mice have been observed at the bottom of the entrance shaft during our visits to the cave.

Final remarks

From a faunistic point of view, Simarrón II is arguably one the most interesting caves in Andalusia and probably in Spain, especially because of the presence of *D. mirabilis* in it. This species was captured (in extremely low numbers, sometimes just one specimen) in the relatively nearby Cueva del Cementerio, Cueva de los Chupones, and Fuente Vieja. Therefore, Simarrón II contains the largest known population to date of this enigmatic carabid species. Its presence in four other locations indicates a broader distribution of the species, although it is still restricted to the southwestern portion of Sierra de Gádor since it was not found in any of the north-facing caves sampled. Instead, another enigmatic endemic troglobite carabid species, *Tinautius exilis* Mateu, 2001 has been described from the caves in the northern slopes of this mountain range. A total of 32 caves have been surveyed in Sierra de Gádor and only 4 of them (12.5%) host *D. mirabilis*; this is remarkable if we consider that other caves are ecologically similar and suitable for this species.

The results obtained in this study suggest that *D. mirabilis* prefers areas of the cave with elevated and stable relative humidity all year round. In Simarrón II, these conditions happen in those chambers farthest from the entrance of the cave, and away from the drastic fluctuation of the exterior: freezing temperatures and snow in the winter, and very hot and dry summers. Even in the stable conditions registered in this study for some chambers of the cave, *D. mirabilis* disappears from the main galleries of the cave and no captures were recorded by the end of the summer. It likely seeks refugee in the micro spaces of the cavern until the first rain arrives at the beginning of the fall season. Besides seasonal changes in the local and the cave climate, the spatial distribution of *D. mirabilis* seems to mainly respond to changes in drought intensity between the cave galleries. This is probably the most important factor triggering the migration of this species and its settlement in a more isolated cave site as Salas Negras. It is far from the influence of the seasonal drops of humidity levels registered in microhabitats nearest to the surface and other well-ventilated areas of the cave. This ecological behavior allows us to consider *D. mirabilis* as a likely indigenous hypogean species that mostly avoids

droughts, accordingly to the classification proposed by Novak et al. (2004). Indigenous hypogean fauna comprises the group of troglobitic and some troglophilous species migrating between the fissure systems and other inaccessible hypogean habitats adjacent to the cave passage and its taxa are very sensitive to constant high level of air humidity.

The results of the current study were presented to the Andalusian Environmental Protection Agency in the South of Spain. These were the base of an initiative to protect this cave and its remarkable fauna. Some of the measures taken by the Administration included the control of human visits to the cave, the installation of a perimetral fence surrounding the entrance (Fig. 1), and the installation of an informative panel at the exterior of the cave describing the endemic entomological fauna it contains. Visits to Simarrón II are only allowed now with a special permit issued by the corresponding Conservation Authorities (Belda et al. 2014). As a result of the studies reported in this work, *D. mirabilis* was included in the list of protected species in Andalusia (BOJA 2012).

Future actions for the conservation of *D. mirabilis* must include a holistic and spatiotemporal study of its populations. This should integrate the conservation and land-use regulation of the entire carbonate outcrop where the four caves with the presence of this species are located, as well as the locally surrounding karst outcrops. In this area of work, although the landscape scale explains better the species composition and distribution, a local scale model in caves suggests the habitat heterogeneity and environmental stability of cave community (as proposed by Ferreira, 2004) as the key parameters to be considered to reliably identify essential features and patterns for conservation and management actions (Pellegrini et al. 2016), for instance when delimiting the species' protection area.

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RESEARCH ARTICLE



Laterality in cavefish: Left or right foraging behavior in Astyanax mexicanus

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Abstract

The evolution of foraging behaviors is key to colonizing challenging habitats such as a cave's dark environment. Vibration attraction behavior (VAB) gives fish the ability to swim in the darkness toward a vibration stimulus produced by many prey crustaceans and insects. VAB evolved in the blind Mexican cave tetra, *Astyanax mexicanus*. VAB is regulated by an increased number of mechanosensory neuromasts, particularly in the eye orbital region. However, VAB in *Astyanax* is only correlated with the number of neuromasts on the left side. *Astyanax* also have a bent skull preferentially to the left and a QTL signal for the right-side number of neuromasts. We conducted field studies in five different cave populations for four years. Results support that all cave populations can express behavioral lateralization or preponderance of side to examine a vibrating object. The percentage of individuals favoring one side may change among pools and years. In one cave population (Pachón), for one year, this "handedness" was expressed by preferentially using the right side of their face. On the contrary, in most years and pools, Tinaja, Sabinos, Molino, and Toro cave populations explored preferentially using their left side. This suggests that if there is an adaptative effect, it selects for asymmetry on itself, and not necessarily for which side is the one to be specialized. Results also showed that the laterality varied depending on how responsive an individual fish was, perhaps due to its nutritional, motivational state, or mode of stimuli most relevant at the time for the fish.

Keywords

asymmetry, Sierra de El Abra, Stygobite, Troglobite, Troglomorphy, VAB

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Introduction

The blind Mexican tetra, *Astyanax mexicanus* (=*Astyanax jordani*), is an emerging model system for the study of evolution. Great advances have been made in understanding the genetic basis of adaptation to an extreme environment, such as living in continuous darkness. The freshwater fish from genus *Astyanax* has two morphs: an eyed surface morph and an eyeless cave morph. The cave morph is restricted to 32 known limestone caves in the Sierra de El Abra region of Northeastern Mexico, and two caves in Guerrero, in Southcentral Mexico (Espinasa et al. 2020). The closely related surface morphs are broadly distributed throughout surface streams and lakes in Mexico.

Throughout their evolution, *Astyanax* cave-dwelling fish have undergone regression of their eyes and pigmentation, whereas the performance of the mechanosensory lateral lines, the olfactory, taste buds, chemosensors, and their foraging behavior have been enhanced to cope with the continuous darkness (Wilkens 1988; Yamamoto and Jeffery 2000; Yamamoto et al. 2004, 2009; Jeffery 2005; Protas et al. 2006; Menuet et al. 2007; Gross et al. 2009; Varatharasan et al. 2009; Yoshizawa et al. 2010; Bibliowicz et al. 2013; Keene et al. 2015; Blin et al. 2018).

The swimming of fish toward an oscillating object, or Vibration Attraction Behavior (VAB) is one of the enhanced behaviors shown in cavefish (Parzefall 1983; Abdel-Latif et al. 1990; Yoshizawa et al. 2010). VAB is mediated by an increase in the cranial superficial neuromasts (Yoshizawa et al. 2010). VAB is aroused in cavefish by vibrations of around 35 Hz (Yoshizawa et al. 2010), which is within the main range produced by many potential prey crustaceans and insects (Lang 1980; Montgomery and Macdonald 1987). The advantage of VAB in foraging has been shown in competitive prey capture assays in the dark (Yoshizawa et al. 2010: Espinasa et al. 2014). VAB appears adaptive in cavefish since it increases foraging ability in an environment devoid of light, with sparse food and few macroscopic predators.

Espinasa et al. (2021) showed that VAB may be present in all cave populations throughout the range of hypogean *A. mexicanus*, spanning from Sierra the Guatemala in the north to southern Sierra de El Abra. However, and most notably, within a given cave the levels of VAB are highly variable between different pools. It appears that individuals inhabiting pools with a high abundance of organic matter have reduced expression of VAB, while in pools with little organic debris, where fish probably depend on hunting more than on scavenging, VAB is enhanced (Espinasa et al. 2021).

Surface morphs are largely symmetric across the left-right axis with little variation (Gross et al. 2015). In contrast, cave-adapted fish have evolved extreme cranial asymmetries. This asymmetry is displayed with a "bend" in their skulls along the anteroposterior axis that is most often biased to the left (Powers et al. 2017). An asymmetric body pattern may negatively affect swimming performance. For example, it could be expected that unequal lateral hydrodynamic resistance (drag) may be energetically costly and result in a preponderance for swimming in circles when visual cues are absent. It can be hypothesized that if cavefish have evolved an extra expenditure due to the drag generated by a bent head, there could be an adaptive compensation from such a unique feature.

Some of the main skull asymmetries are found in the dermal bones that encircle the eye orbit and, in particular, on the third suborbital (SO3) bone, which is commonly fragmented in an irregular architecture across the lateral cranial complex (Gross et al. 2014). Tactile sensitivity in cavefish is mediated by the lateral line, a sensory system comprised of mechanosensory neuromasts. The superficial neuromasts distributed within the eye orbit region of the face are the most important in mediating VAB (Yoshizawa et al. 2010). However, further analyses by Fernandes et al. (2018) showed lateralization; cavefish from Pachón cave show a positive correlation of VAB with the number of neuromasts on their left side but not on their right. This laterality was not seen in *Astyanax* surface fish under any conditions tested, suggesting this laterality emerged through an evolutionary process. They concluded that *Astyanax* cavefish have higher developmental plasticity in neuromast number and bone formation and that cavefish showed an asymmetric correlation between the number of left-right neuromasts and VAB.

Subsequent studies have shown that neuromasts placement and suborbital bone formation are linked during development and that canal neuromast position prefigures developmental patterning of the suborbital bone series in *Astyanax* cave- and surfacedwelling fish (Powers et al. 2018). Using a QTL approach, Powers et al. (2020) showed that the genetic architecture of neuromasts present in the eye orbit region is under genetic control with a lateral asymmetry; linked loci signals were detected for the right side of the face, but not for the left side. The locus for neuromast position maps near established QTL for eye size and facial bone morphology, suggesting that eye loss, sensory expansion, and the cranial skeleton may be integrated for evolving adaptive behaviors. It should be pointed out that while there is a QTL signal for the number of neuromasts only on the right side of the face, the positive correlation of VAB is with the number of neuromasts on only their left side. Both studies used lab stocks derived from Pachón cave (QTL specimens were derived from specimens originally collected by Dr. Richard Borowsky of New York University, while the ones for the VAB studies were collected by Dr. William Jeffery of The University of Maryland).

These findings, a bent skull preferentially to the left, a correlation of VAB with left side number of neuromasts (in Pachón cave), and a QTL signal for the right-side number of neuromasts, suggest laterality in enhanced sensitivity for different modes of stimuli and the potential for behavioral laterality with different side swimming preference, or handedness, for different behaviors. While there is no preponderance for swimming direction in familiar settings in the laboratory, cavefish from Pachón cave prefer to use their right-side lateral line when encountering a novel stationary landmark (De Perera and Braithwaite 2005) but until now, it is unknown if there is a side preponderance for any foraging behavior including VAB.

In hypothesizing for an adaptive reason for a curved or bent skull, we first tested if the skull or the body of *A. mexicanus* had a size that could theoretically reflect 30 and 40 Hz waves to amplify a signal, similar to a parabolic amplifier. To work as a parabolic amplifier for waves, the corresponding parabola size must be of the wavelength order or bigger (Rumsey and McCormick 2006). We then tested whether there is laterality in response to a vibrating stimulus in their natural habitat. Considering that different cave populations may have different evolutionary histories and that the expression of VAB can differ even between pools of the same cave, field research was conducted on 11 different pools in five caves encompassing the Sierra de Guatemala and the Sierra de El Abra, on four different years.

Methods

In the water, potential prey can emit both sound waves and ripple waves. To test if the curved shape of a fish while circling a vibrating stimulus or the bent shape of a cavefish skull have the minimum theoretical size to reflect either type of waves in such a way as to focus wave signals, the following formulas were used. Wavelength λ can be computed if propagation speed v_{phase} and frequency v are known: $\lambda = v phase/v$.

Sound (pressure waves) travels through water at a speed close to 1,480 m/s. Yet, surface ripples travel much slower in deep water (depth > λ). In a previous study (Espinasa et al. 2021), the speed of ripples on the water surface of the actual pools inhabited by cavefish was measured for 35 Hz frequencies, with results between 10.7 and 17.2 cm/s. With these results, a plot was made for wavelengths versus phase velocities to determine the theoretical minimum size of cavefish structures that could serve as amplifiers.

For the field experiments, the care and use of experimental animals complied with Mexican animal welfare laws, guidelines, and policies Fieldwork Mexican permits 02438/16, 05389/17 and 1893/19 (Patricia Ornelas-Garcia and LE) were delivered by the Secretaria de Medio Ambiente y Recursos Naturales. The cave populations studied were Molino, Pachón, Sabinos, Tinaja, and Calera (within the Toro cave system). The first is located in the Sierra de Guatemala and the rest in the Sierra de El Abra, all in Northeast Mexico. For a full description of the caves, their topography, localization, and ecology, see Elliott (2018) and Espinasa et al. (2020).

An apparatus for delivering vibrations was obtained and modified to meet the requirements for waterproofness, portability, and reliability needed for the trying conditions of fieldwork in a cave. A vibrator device connected to a potentiometer to control the amount of current and thus speed control was used (Espinasa et al. 2021). Calibration and corroboration of the vibration frequency emitted by the apparatus were done as in Espinasa et al. (2021). The average value of the actual frequencies that were delivered in the field in multiple pools was 35.5 Hertz, with a standard deviation of 6.29, a minimum frequency of 31.5, and a maximum frequency of 42.6. The video analyses started only a minimum of 5 sec after the device entered the water to reduce the effect of the sound of the device first entering the water. The process was repeated alternatively with the vibrator on and off. Results for the difference between on and off can be found in Espinasa et al. (2021); when vibration is on, fish swim towards the device, and then highly responsive individuals spin in circles around the device, oftentimes biting it.

Video recordings were done in February of 2022 of two pools from Pachón cave and one pool from Tinaja cave. Pachón's "Large pool" is the one first encountered when entering the cave and 31 recording were performed there. 150 m further in, there is the "Maryland pool" in the Maryland extension portion of the cave and 56 recordings were performed here. Tinaja's "4th pool" is found deep into the cave in the section dubbed "Sandy Floored", and 21 recording were done. When the first fish circled once around the vibrating device, filming was stopped and the device was taken out of the pool. The individual fish that conducted the behavior was captured with a hand net and deposited in an inflatable pool. This guaranteed that data for statistical analyses corresponded to single events conducted by different fish within the population. Vibrating device was not put back into the water until about 5 minutes later. Filming was performed with a DCR-SR42 Sony Digital camera. VLC Media Player was used to analyze the collected videos. The expected proportion of fish performing left-side turns versus right-side under the null hypothesis is 0.5. Statistical significance was determined using the binomial test (The non-parametric X² test with a Yates correction for continuity [Yates 1934], as recommended when v = 1 [Zar 1984] gave equivalent and corroborating P values).

We were also interested in analyzing behavior in other caves and across multiple years. For this, video clips produced by Espinasa et al. (2021) were used, giving data for comparisons among five different caves and 11 pools over four different years. When those videos were taken, the purpose of the study was simply to determine the presence or absence of VAB. Therefore, the vibrating apparatus was left in the water for extended periods and fish that had performed a circling behavior were not extracted. Therefore, individual fish could have engaged in multiple circling episodes and be present in multiple video clips. Due to the inability to discriminate if single individuals were responsible for repetitive events in the data, a different statistical analysis was used that could account for this limitation; different pools and years were counted as independent observations, so Pachón and Tinaja have an N = 7 each and Sabinos an N = 3. The number of left-side turns versus right-side turns was tested by fitting generalized linear mixed-effects models by using the binomial distribution (the Beta-distribution test gave equivalents and corroborating P values). Molino and Calera cave populations each had an N = 1, so no statistical analysis was performed. Examples of the video clips that were used by Espinasa et al. (2021) for Pachón cave can be seen at https://www. youtube.com/watch?v=RAvuH2MM0z8, and for Tinaja cave at https://www.youtube. com/watch?v=C0Zz7g4RChk.

Fish attracted to the vibration device in the video clips from Espinasa et al. (2021) show two types of behavior; they can swim towards the device and then swim away, or they can be highly responsive, swimming in circles around the device. Two types of data gathering were performed to discriminate highly responsive fish from the overall response of the population. To measure the side preponderance of the highly responsive specimens, all individual fish were tracked by one of the authors of this paper using the VLC Media Player to see if they circled the vibrating device clockwise or counter-clockwise. When a fish circled the vibrator device for at least 270 degrees around the

device, showing at all times the same side of the face, it was counted as the first turn around the device. If the individual fish continued circling the device two, three, or more times, the number of turns was counted subsequently from the initial starting point. When a fish changed its path, so the other side of the face was towards the device, the number of turns for that side of the face stopped, and counting was restarted.

To analyze the overall response of both highly responsive and less responsive fish, the videos were divided into 5-second clips, and the frozen image at the end of each clip was analyzed. The number of fish inside a 6.25 cm radius circle in the still image was counted, as in Espinasa et al. (2021), but this time dividing the number of individuals facing the apparatus with their right side or left side of the body. Fish analyzed with this system included both the highly responsive fish that circled the device and the less responsive that swam away after approaching the device.

Previous VAB studies conducted in the laboratory (Yoshizawa et al. 2010) have been performed under the illumination of infrared light (880 nm wavelength, BL1960-880 blacklight, Advanced Illumination). As described in Espinasa et al. (2021), we attempted to use the Night Vision feature of the DCR-SR42 Sony Digital camera to record behavior in the field. Regrettably, it is of insufficient intensity to effectively be used in the trying conditions of the field. Muddy banks or pools at the bottom of small pits often did not allow the camera to be positioned close enough for the night vision illumination. It was found that the night vision illumination barely penetrated underwater, and fish could not be accurately seen. Videos analyzed were the same as those used in Espinasa et al. (2021), where illumination with standard headlamps (Sten light) was used to record when the vibrator was off and when it was on. As discussed in Espinasa et al. (2021), since the light was used both when the stimulus was absent and present and for fish using their left side and right side, it is not an experimental variable. The eyeless fish handedness response to the vibration stimulus was determined for both sides under identical illumination conditions.

Results

Our first hypothesis was that the curved body shape of *Astyanax* fish while turning around a vibrating stimulus, or the bent shape of a cavefish skull, has a size that allows them to efficiently reflect water waves with a function akin to a parabola (Fig. 1A, B). When we tested through mathematical modeling for sound (pressure waves) that travel through water at a speed close to 1,480 m/s. Results show that the corresponding theoretical wavelengths for 30 and 40 Hz used for VAB are 50 and 37 m (Fig. 1C). It is very unlikely that a small fish body (~ 10 cm) can work as a parabolic amplifier for sound at those wavelengths.

Yet, ripple waves are not pressure waves, and their mechanical manifestation is mainly by water flow, where fluid particles are in an orbital motion. This motion diminishes strongly with depth, so ripple waves stay very close to the surface, and have much slower speeds (Feynman et al. 1963). When modeling wave frequencies of 30–40 Hz



Figure 1. A schematic depiction of a plane wave (rays) reflected on the fish's body. In this case, the waves are being amplified towards the head neuromasts. The black dotted lines represent tangents to the fish lateral line at the reflection points **B** deflection of plane waves on the bent skull of a cavefish **C** wavelength is plotted against every phase velocity between 2 cm/s and 10,000 m/s (logarithmic scale). The blue shade aims to highlight the wavelength region for which parabolic amplifiers can work in a 10 cm long fish. Clearly, the speed of sound (1,480 m/s) is off limits for frequencies at around 35 Hz, but surface ripples that travel at 10.7–17.2 cm/s are within the proper wavelength range to be efficiently reflected by structures of a minimum length of 0.2–0.4 cm. Thus, curved bodies of a fish when circling around a vibrating stimulus or a bent skull can theoretically serve as amplifiers for the ripple waves generated by prey, but not for the sound that prey also produce. Skull images modified from Gross et al. (2014).

and a speed of 10.7–17.2 cm/s, which is the actual speed of ripples on the surface water of the pools inhabited by the cavefish as determined by Espinasa et al. (2021) results from mathematical modeling (Fig. 1C) showed that the theoretical minimum size for a surface to efficiently reflect this type of waves is between 2–4 mm. Thus, the length of an adult *Astyanax* and the size of their bent skulls are within the theoretical ranges to efficiently deflect this type of waves towards the main superficial neuromasts responsible for mediating VAB.

Our second hypothesis was whether there is laterality in response to vibrating stimulus in their natural habitat. In the field, cavefish responded to the vibrations generated by approaching the device using the neuromasts of the left side of their face or their right side. Highly responsive individuals then circled the device counterclockwise



Figure 2. Cavefish explore a vibrating apparatus by circling counterclockwise (T1–T4 left), using their left side, or clockwise (T1–T2 right), using their right side. T1–T4 are sequential images from a video clip with arrows following the path of single fish while circling. Graph shows that preponderant side with which cavefish circle around a vibrating stimulus is significantly different between cave populations (Brackets for 90% confidence) **A**, **B** skull images modified from Gross et al. (2014) showing that the left bending of the skull is different between Tinaja and Pachón cavefish.

(Fig. 2 T1–T4 left) or clockwise (Fig. 2 T1–T4 right). When the device was off, all populations showed no right side or left side preponderance (all populations P > 0.35). When the device was vibrating, data shows that cave populations have a laterality or preponderant side with which to explore the source of vibrations (Figs 2, 3), But this laterality can be different among different caves: at Tinaja's 4th pool preponderance was for the left side (15 Left; 6 Right), while at Pachón's large pool (9 Left; 22 Right) and at Maryland's pool (15 Left; 41 Right) the preponderance was for the right side, as seen in figure 2 (Tinaja vs. Pachon's large pool P = 0.00641; Tinaja vs. Pachon's Maryland pool P = 0.00091). When analyzing only the highly responsive individuals that engaged in circling behavior, most populations showed a higher proportion of left turns (Figs 3, 4). In the Molino cave population, 80% (16 left vs. 4 right) of the turns were in a left direction. In Sabinos, 94.4% (34 left vs. 2 right; P = 0.00096). In Tinaja, 73.0% (134 left vs. 50 right; P = 0.00675). In Calera, 72.4% (119 left vs. 44 right). No P values are offered for Molino and Calera since their N = 1. Only at Pachón cave did the fish show a higher number on for the opposite right turns (36.6%; 100 left vs. 173 right; P = 0.06051).

The border-line of a P = 0.06051 from the Pachón population for not significantly differing from a 1:1 ratio warranted further analyses. In 2022, the data collection method was improved to eliminate the effect of individual fish performing multiple circling and their presence in multiple video clips. With this improved method, at



Figure 3. Preponderance in circling a vibrating stimuli using the left side or right side in different cave pools and times. As a population and throughout time, Sabinos and Tinaja cavefish used significantly more their left side. When all years and pools are included, Pachón showed a border line P = 0.0605 (in green) for not significantly differing from a 1:1 ratio. N's in upper row are for all four years. But when the enhanced data collecting method was used in 2022, fish from two Pachón pools significantly used their right side more. Videos from 2017–2020 are from Espinasa et al. (2021). For the recordings of 2022, methodology was improved such that it guaranteed that data was from independent fish within the population, allowing for statistical analyses for that single pool and date.

Pachón's Maryland pool, only 26.7% (15 left vs. 41 right; P = 0.00083) of the fish that circled were in a left direction, and in the large pool, 29.0% (9 left vs. 22 right; P = 0.03114).

For some caves, the preponderant side preference was relatively high among pools and in different years (Fig. 3). It is known that both Sabinos and Tinaja caves are part of a single hydrologic system (Elliott 2018), where cavefish can probably move from one cave to the other in extreme rainy seasons. Out of ten measurements in these two caves from four years of observations in a total of six different pools, only once did leftpreponderance not dominate in their response to VAB. But it appears that side preponderance can also change over time and among pools. For Pachón cave (Fig. 3), four data points hovered around a 1:1 ratio, but in three, there was a preponderance for the right side (As mentioned above, two of them were significantly different from a 1:1 ratio).

The above results were for only highly responsive fish that circled the vibrating apparatus. As described in the methods, we did a second type of analysis that includes less responsive fish that just approached the device and then swam away, plus the responsive fish that circled the device. In this case, an intriguing trend was found for all populations: highly responsive fish appear to proportionally use their left side more



Figure 4. Highly responsive fish tend to proportionally use their left side more when compared to those individuals that display a less responsive behavior towards a vibrating stimulus. "# of fish" includes both individuals that only approach the device and then swim away and highly responsive fish that circle around the device. "Turns" counts the behavior exclusively of the highly responsive fish that circle the device. It would appear that highly motivated fish enhance their left-sidedness in their foraging behavior when responding with VAB, except perhaps in Pachón cave population.

when compared to less responsive individuals (Fig. 4). At Molino cave, right side use decreased from 39.1% to 20% when comparing data that includes less responsive fish that approach and then leave without circling versus only fish that circle. Similar decrease of right-side use was seen in Sabinos (26.2% to 5.6%), Tinaja (31.7% to 27.0%), Calera (38.1% to 27.6%). In other words, highly responsive fish seem to increase their left side preference. Only in Pachón cave did there appear to be no to minimal change (60.1% to 58.9%).

Discussion

Our results show that the head and body of *Astyanax* cavefish are within the theoretical size range to serve a function akin to a parabola for ripple waves being amplified for VAB, but not for sound (Fig. 1). Potential prey makes both sound waves and ripple waves. Both signals may be used by cavefish. What we have shown is that if there is an adaptive reason for the bent skull, it is unlikely that it is for amplifying sound waves, but it is at least theoretically possible that it is for amplifying ripple waves. Future

studies may resolve if ripple waves are actually being deflected in such a way that it amplifies the signal and if the bent skull shape of cavefish is implicated.

An alternative for the origin of a bent skull is that what is being selected is the neuromast number or properties, and suborbital bones are just developmentally linked. Powers et al. (2018) have shown that the canal neuromast position prefigures developmental patterning of the suborbital bone series in *A. mexicanus* fish. It may be that there is a selective advantage to asymmetric neuromast and the skull gets bent through pleiotropy or linkage.

Apart from a bent skull predominantly to the left (Powers et al. 2017), previous studies have shown that there is a correlation of VAB with left side number of neuromasts in Pachón cave population (Fernandes et al. 2018), and a QTL signal for the right-side number of neuromasts (Powers et al. 2020). These literalities suggest the possibility of swimming side preference for different behaviors to enhance sensitivity depending on the types of stimuli. It is known that cavefish prefer to use their right-side lateral line when encountering a novel landmark (De Perera and Braithwaite 2005), but before this study, it was unclear if there was a side preponderance for any foraging behavior including VAB.

We tested for laterality in response to VAB in the field and our data supports that all cave populations expressed laterality and significantly differed from an expected 1:1 ratio on the side with which they circle a potential prey, at least in some years and for some pools (Fig. 3). Astyanax populations from four out of the five caves studied (Molino, Sabinos, Tinaja, and Toro system) preferentially circled with their left side of the face towards the source of vibrations. In some years, subpopulations in several of Pachón's cave pools had no statistically significant lateral preponderance, but at other times they showed a significant preponderance for using their right side towards the stimulus (Figs 2, 3). It remains to be tested if VAB differences in preference of side between these cave populations are due to environmental conditions, skull differences, neuromast differences, or other genetic reasons. One hypothesis could be that in evolving for foraging in the darkness, laterality is enhanced through asymmetric sensitivity for different modes of stimuli or lateral swimming preference. Yet, the side itself may be irrelevant. It may well be that evolutionary histories, adaptive constraints, or stochastic circumstances allowed Pachón's population to have percentages of individuals that have left-preference or right-preference different from other caves and perhaps variable at different times.

Espinasa et al. (2021) showed that VAB expression could be variable within a single cave and among pools and found it to be correlated to environmental variables such as the amount and type of available food. It would appear that individual fish may change their expression of VAB according to their physiology, motivation, and nutritional state. Our results suggest that the proportional use of sidedness is also modified depending on the responsiveness of individual fish. It may be that fish that are hungry and more motivated to search for food rely differently on specialized sensory mechanisms affecting sidedness. One could expect differences between pools where most food consists of guano and pools with mostly vibrating prey. Support for this notion can be found by comparing subpopulations in different pools at Pachón cave. Espinasa

et al. (2021) noticed that Pachón's large pool contained low levels of organic carbon while the small pool, which has a small bat roost on top, had a significantly higher level of organic carbon. Our results show that both in 2019 and 2020, the large pool, which presumably has a lower density of guano-derived nutrients, had proportionally higher laterality favoring the right side than the smaller pool, which has higher organic densities (See fig. 3; 2019 = 53.0% left in the small pool vs. 47.1% left in the large pool; 2020 = 57.1% left in the small pool vs. 19.2% left in the large pool).

Caution should be used when evaluating results in this and future studies since the preponderance for laterality during foraging behavior may fluctuate due to environmental conditions and over long periods of time. The studies by Gross et al. (2015) and Powers et al. (2017) have shown that in laboratory-bred cavefish, there is variability in how bent the skull is. While the majority have a left bent in both Pachón- and Tinajaderived fish, some individuals can have a right-bent skull. A limitation of behavioral studies with laboratory stock-fish is that they are derived from a few field-collected specimens that have been bred for several generations, and some bottleneck effects can be expected. Laboratory animals could be expressing a preponderance in side of their behavior different from the one currently being expressed in the field. For example, Espinasa et al. (2021) showed that while some laboratory stock populations derived from Tinaja and Molino were reported as VAB reduced (Yoshizawa et al. 2010, 2015; Yoshizawa 2016), in the field, those same caves have abundant VAB positive individuals. Likewise, Pachón laboratory stock animals are derived from field collections done before 2017. In our data, Pachón population did not show clear laterality in 2017 and 2019, but after 2020 subpopulations had significant VAB laterality.

In conclusion, while previous studies have shown that cavefish from Pachón cave prefer to use their right-side lateral line when encountering a novel stationary landmark (De Perera and Braithwaite 2005), this is the first time that it has been shown that *Astyanax* cavefish show laterality in their foraging behavior in the field. Likewise, we have shown that a cavefish's skull is of a size that at least theoretically could serve as an amplifier, thus allowing for its bent shape to have an adaptive function.

Conclusions

1) If VAB mediating neuromasts are more proficient on one side of the head, our theoretical analyses have shown that the size of a bent skull or of a whole-body flexed when circling a vibrating stimulus is sufficient to provide an efficient surface on which to reflect and concentrate wave signals towards that spot (Fig. 1).

2) *Astyanax* cavefish show laterality in the preponderant side they use to explore vibrating stimuli (Fig. 3).

3) Which side is preponderant can vary between different cave populations (Fig. 2).

4) Laterality towards VAB may be plastic; how responsive or motivated a particular individual is to respond towards a vibrating stimulus can have an effect on which side they preferentially use (Fig. 4). 5) We have found some degree of variability in the expression of laterality among pools, and throughout time within a single cave. The proportion of individuals within a population that have a certain lateral number of sensory neuromasts, bending of their skulls, and/or any other characters that may be involved in lateralization of foraging behavior may change due to stochastic effects (bottlenecks) and/or natural selection due to local environmental conditions.

6) Caution should be used when comparing VAB and laterality results. Nutrition levels in the different pools in the field and the laboratory may be drastically different. Likewise, acclimatization to preferentially eat live prey, guano, or even pet food throughout their development may accustom individuals to use different types of receptors.

Significance statement

Vibration attraction behavior (VAB) confers the ability to swim toward water disturbances generated by prey in the darkness. VAB has a genetic component and evolved in cavefish. Cavefish also have a unique asymmetry of a "bend" in their skulls and a correlation of VAB only with the left side number of neuromasts. An asymmetric body pattern may negatively affect swimming performance and create an unequal lateral hydrodynamic resistance (drag). A possible explanation is that as an adaptive compensation, lateralization may enhance the dynamics of water signaling, signal reception, or signal processing on one side. Our results support this association in showing there is laterality in VAB's response, where a vibrating object is examined preferentially with one side of the face. However, which laterality, right side or left side, appears to be variable among different cave populations. Perhaps the adaptative nature of this phenomenon selects for asymmetry by itself and does not necessarily select for which side is the one to be specialized.

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RESEARCH ARTICLE



A new species of the genus Siambathynella Camacho, Watiroyram & Brancelj, 2011 (Crustacea, Bathynellacea, Parabathynellidae) from a Thai cave

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Abstract

Siambathynella janineana **sp. nov.** (family Parabathynellidae) discovered in Thailand is herein described. The new species displays an exclusive character: a distinctive organ on the antennule of males. It also displays a unique combination of morphological characters, including a seven-segmented antennule and antenna, sexual dimorphism of the antennule, a one-segmented exopod on thoracopod I and a two-segmented one on thoracopods II to VII, a male thoracopod VIII without basipodial setae and with a trapezoidal outer lobe, a female thoracopod VIII with two denticles at the base of two setae, pleopods absent, a sympod of the uropod with an inhomonomous row of spines and an uropod endopod with a spinous distal process and a very large distal spine covered with setules.

This is the fifth record of Bathynellacea (family Parabathynellidae) found in caves in Thailand.

Keywords

cave, groundwater, Parabathynellidae, Siambathynella, Thailand

Introduction

The family Parabathynellidae is currently comprised of 45 genera and 220 species worldwide (210 species, Camacho et al. 2021a, b, 2022). Of these known species, around 77 belonging to 16 genera occur in Asia (Camacho 2019). To date, most

of the Asian species that have been described are from South Korea (27 species) and India (23 species), with very few species known from Thailand and nearby countries. Only two genera (and four species) belonging to the Parabathynellidae have been described from four caves in Thailand: Paraeobathynella Camacho, 2005 (three species) and Siambathynella Camacho, Watiroyram & Brancelj, 2011 (one species). This last genus is characterized by the presence of a male antennal organ, an uncommon trait in Parabathynellidae. Indeed, only six of the family's genera have species that display this type of sexual dimorphism (Camacho 2019). Three of the genera with species characterized by a male antennal organ, though with varied morphology, are found in Asia: Siambathynella (Siambathynella laorsriae Camacho, Watiroyram & Brancelj, 2011) and some Indian species of Chilibathynella Schminke, 1973 (C. kotumsarensis Ranga Reddy, 2006) and Atopobathynella Schminke, 1973 (A. operculata Ranga Reddy, Drewes & Schminke, 2008; A. paraoperculata Ranga Reddy & Totakura, 2015; A. indica Bandari, Shaik, Ranga Reddy & Totakura, 2017; A. nelloreensis Bandari, Shaik and Ranga Reddy & Totakura, 2017 and A. inopinata Bandari, Shaik, Ranga Reddy & Totakura, 2017). Hexabathynella Schminke, 1972, a cosmopolitan genus of Bathynellacea, contains 23 species whose males present a highly complex antennal organ not found in females (Cho and Schminke 2006); however, none of these species occur in Asia. Siambathynella is the only genus found in Thailand that shows sexual dimorphism in article two of the antennule. Here, we describe the morphology a new species of Siambathynella, Siam*bathynella janineana* sp. nov., the second species of the genus occurring in Thai caves. The new species undoubtedly belongs to Siambathynella as it shows a combination of morphological features representative of the genus and some exclusive characters.

Siambathynella laorsriae Camacho, Watiroyram & Brancelj, 2011 was found in a cave in the northern of Thailand (Nam Nao National Park, Phetchabun) as the new species described here (Chiang Dao District). The other genus present in Thailandia, *Paraeobathynella*, is represented by the species, *P. ratensis* Camacho & Watiroyram, 2018, *P. siamensis* Camacho & Watiroyram, 2018 and *P. hanjavanitiana* Camacho & Watiroyram, 2018, that live in three tourist cave located in the Nakhon Si Thammarat mountain range (Thung Song District) in the southern of Thailand.

Material and methods

Study area and groundwater sampling methods

In 1985, a French-led expedition comprised of the biologists and speleologists A. Bedos, L. Deharveng, J. Gibert, B. Lebreton and P. Leclerc carried out explorations and sampling of caves in Thailand and Indonesia (Zeitoun et al. 1985). One of the explored cavities, Tham Chiang Dao, Amphoe Chiang Dao, Chiang Mai Province, Chiang Dao District, Northern Thailand (coordinates: 19°23'38"44°N, 98°55'41"00°E – Z 455 m), is the type locality of the new species. This cave is located in one of the largest limestone regions in the world, which includes most of southwestern China and a part

of northern Vietnam, extending through the Golden Triangle, i.e., the meeting point of the borders of Laos, Thailand and Burma (Myanmar). The region is characterized by a type of humid tropical karst with a long pre-quaternary evolution: the morphology is that of an "inselberg" karst (Gibert 1987). This type of karst develops in the primary Permian limestones of the Ratburi formations. Most of the cave is fossil and opens at the level of the alluvial plain by an exsurgence on carboniferous grounds comprised of cherts, sandstones, schists and conglomerates (Gibert 1987). The specimens used for the description of *Siambathynella janineana* sp. nov. (14 males, 21 females and one juvenile) were sampled from a medium-sized muddy pool (mainly, colloidal silt) located in the upper fossil gallery of the cave. The sampling was carried out by removing the sediment from the bottom of the pool and using a hand-held plankton net with a 0.112-mm mesh to filter specimens from the murky water. The collected specimens were preserved in 70% ethanol. Given this preservation method and the length of time since collection, we were unable to extract DNA from the specimens for complementary molecular studies.

Morphological study

Of the 36 specimens sampled,16 (8 males and 8 females) were processed for the detailed morphological study. Four specimens (2 males, 2 females) were mounted *in toto* and 12 were completely dissected (i.e., all body appendages were separated) and preserved on permanent special metal slides (with glycerine-gelatin stained with methylene blue and paraffin as the mounting medium) as previously described by Perina and Camacho 2016. Anatomical examinations were performed using a Zeiss interference microscope fitted with an oil immersion lens (100×) and a drawing tube. Material were deposited in the Arthropod Collection held at the Museo Nacional de Ciencias Naturales (MNCN) in Madrid, Spain. The terminology adopted was as in Serban (1972) and Schminke (1973).

Abbreviations used in the text and figures

Morphology

AI = antennule; AII = antenna; Bsp.= basipod; D.Lb.= dentate lobe; Endp.= endopod; Exp.= exopod; I.Lb.= inner lobe; Lb = labrum; Md = mandible; MxI = maxillule; MxII = maxilla; O.Lb.= outer lobe; Sgt = article; ThI–VIII = Thoracopods I–VIII; Symp = Sympod; Urp = uropod.

Acronyms

MNCN	Museo Nacional de Ciencias Naturales de Madrid, (Spain).
CSIC	Consejo Superior de Investigaciones Científicas (Spain).
ARTP/MNCN	MNCN Arthropod Collection.

Results

Systematic account

Order Bathynellacea Chappuis, 1915 Family PARABATHYNELLIDAE Noodt, 1965

Genus Siambathynella Camacho, Watiroyram & Brancelj, 2011

Amended genus diagnosis. after Camacho, Watiroyram and Brancelj, 2011.

Antennule seven-segmented, without aesthetascs on the fifth article and with subterminal aesthetascs on the last article, sexually dimorphic, with a male antennal organ on the second article of the antennule. Antenna seven-segmented, first, second and fifth articles without setae. Mandible with expanded distal claw on *pars molaris*. Maxilla with four articles. Exopod of thoracopods II to VII each two-segmented, that of ThI one-segmented; basipod of thoracopods I to VII with one smooth seta; epipod of ThI absent. Male thoracopod VIII: almost square, with small endopod integrated into basipod and one or two smooth long setae; inner lobe incorporated into basal region, slightly shorter than dentate lobe; basipod very large, almost square and with a row of small denticles on the distal part, proximal side with two lobes, one of which is curved inwards and almost completely covers the exopod, without setae. Female thoracopod VIII: with two long setae. Pleopods absent. Ventro-lateral seta of pleotelson located adjacent to the insertion of the furca. Inhomonomous uropod sympod with a row of spines occupying the distal half; endopod with a spinous projection on the distal outer corner and with one thick spine covered with setules.

Type species. Siambathynella laorsriae Camacho, Watiroyram & Brancelj, 2011.

Siambathynella janineana sp. nov.

https://zoobank.org/F4366538-281F-40A4-92E5-6BAEE6D3E3E2 Figs 1–4

Type locality. Tham Chiang Dao Cave $(19^{\circ}23'38"44^{\circ}N, 98^{\circ}55'41"00^{\circ}E - Z 455 m)$, Thailand. Collected by Philippe Leclerc (17/07/1985).

Type material. *Holotype* male (MNCN 20.04/20552), allotype female (MNCN 20.04/20553) and type series comprised of seven males (MNCN 20.04/20554 to MNCN 20.04/20560) and seven females (MNCN 20.04/20561 to MNCN 20.04/20567), each on individual slides.

Description. *Body* (Fig. 1). Total length of holotype 1.25 mm; allotype 1.5 mm; male length, 1.1–1.6 mm; female length, 1.0–1.8 mm. Body elongated, segments slightly widen towards posterior margin; head about as long as broad. Pleotelson with one plumose ventro-lateral seta on each side. All drawings are of the (male) holotype except for the female ThVIII and second article of the antennule, which were drawn from the female allotype.



Figure 1. Siambathynella janineana sp. nov. Habitus male.

Antennule (Fig. 2A). Seven-segmented; first three articles slightly longer than the other four; first two articles similar in length and the third is the longest; sexual dimorphism manifested by the presence of a well-developed antennal organ on the inner distal margin of the second article in males (Fig. 2A), organ represented as a protrusion with a smooth, moderately long seta in its middle lateral area. On the female antennule, only a short seta is present in the homologous position as the male antennal organ (Fig. 2C); inner flagellum almost square; fourth article slightly longer than the fifth, with two aesthetascs, which is the smallest of all the articles; sixth and seventh articles each with three aesthetascs, which are placed sub-terminally on the last article; setation pattern as shown in Fig. 2A.

Antenna (Fig. 2B). Seven-segmented; approximately 70% the length of AI; first three articles small, all similar in size to the fifth and about 50% the length of the fourth article, which is similar in size to the last two articles; distal article with four setae, one of which is plumose. Setal formula: 0/0/1+0/1+1/0+2/4(1).

Labrum (Fig. 2D). Almost flat with eight main teeth, with the two central teeth that are slightly different from the others, more rounded, plus two lateral teeth on each side. Ventral surface ornamented with rows of fine spinules.

Mandible (Fig. 2E). *Pars incisiva* with four teeth and a well-developed triangular tooth on the ventral edge; *pars molaris* with five claws, one strongly modified distal claw that is slightly separated from the rest, with small denticles, and four claws (the two proximal ones are joint) with fine long setules; mandibular palp one-segmented, with a distal seta that does not exceed the length of the *pars incisiva*.



Figure 2. *Siambathynella janineana* sp. nov. All drawings of the male holotype except in **C**. **A** AI **B** AII **C** second article of AI of female allotype **D** Lb **E** Md **F** MxI **G** MxII **H** antennal organ on second article of AI in *Siambathynella laorsriae* Camacho, Watiroyram & Brancelj, 2011. Scale bars in mm.
Maxillule (Fig. 2F). Proximal endite with four long serrulate claws; distal endite with seven claws, the basal one very thin, the two distal ones smooth and the other five with denticles, and with three sub-terminal smooth setae on the outer distal margin.

Maxilla (Fig. 2G). Four-segmented, first two articles with an elongated endite, with two and four setae, respectively, one setae on each endite plumose; third article rectangular with one strong claw and eight smooth setae; fourth article reduced with five setae. Setal formula: 2, 3+1, 9, 5.

Thoracopods I–VII (Fig. 3A–G). Well developed, gradually increasing in length from ThI to ThV, last two thoracopods similar in length; ThI without epipod, ThII–VII with large epipod, each about the same length as the corresponding basipod; basipod of ThI–VII with one smooth seta on inner distal corner. Exopod of ThI one-segmented, two-segmented in ThII–VII; exopod longer than first two endopod articles in ThII–VI and equal in length in ThVII; two barbed setae on each exopodal article and with one group of strong ctenidia at the base of the inner setae. Endopod four-segmented; first article short, second and third long and similar in length, both with a cluster of strong spinules along the outer margin; fourth article reduced, with two smooth, strong claws of similar length and one seta; pair of smooth inner setae on first article present only on ThI; inner setae on article two always barbed; outer small seta on third article of ThI–VII smooth; outer distal setae on second article of all Ths plumose. Setal formula of endopod as follows:

Thoracopod I	2+0/2+1/1+1/3(1)
Thoracopod II	1+0/2+1/1+1/3(1)
Thoracopod III and IV	0+0/2+1/1+1/3(1)
Thoracopod V to VII	0+0/1+1/1+1/3(1)

Thoracopod VIII (Fig. 4A, B). Almost square; basal region of the penial complex with three lobes: inner lobe (I.Lb.), outer lobe (O.Lb.) and dentate lobe (D.Lb.); rectangular inner lobe completely integrated into basal region, a little shorter than dentate lobe; trapezoidal outer lobe as dentate lobe; endopod (Endp.) integrated on basipod, with one long smooth seta; reduced exopod, with two spinules; basipod very large, square, slightly recurved caudally, with a distal row of small denticles, with two lobes on the frontal side, one of which is recurved inwards (crest-like protuberance), without setae. The ThVIII of the type species, *S. laorsriae*, is shown for comparison (Fig. 4C).

Thoracopod VIII female (Fig. 4D). One-segmented with two long smooth setae and two small denticles at the base.

First pleopods. Absent.

Uropod (Fig. 4E). Sympod five times as long as wide and twice as long as endopod, with nine barbed spines about equal in length except the distal-most, which is twice as long as the others, occupying the distal half; exopod 30% longer than endopod, with four barbed setae, two terminally and unequal in length; distal outer corner of endopod forming a spinose process, with two row of setules and one strong spine that is 20% longer than the endopod, with setules, one plumose seta near the base and two small barbed terminal setae of different lengths.



Figure 3. Thoracopods I to VII of *Siambathynella janineana* sp. nov. Male holotype **A** ThI **B** ThII **C** ThIII **D** ThIV **E** ThVI **G** ThVII. Scale bars in mm.

Pleotelson (Fig. 4F). With one small, plumose ventro-lateral seta on each side close to the insertion of the furca. Anal operculum slightly concave.

Furca (Fig. 4F). Rectangular, with three barbed spines, outer two spines twice as long as the innermost one; two equally long dorsal plumose setae; lateral furcal organ "cork"-like.

Variability. The number of spines on the uropod sympod varies between nine and 12, with the larger specimens showing a higher number of spines. Variation is also observed in the setal formula of the endopod of the thoracopods: ThI, 2+0/2-3+1/1-2+1/3(1); ThII, 1+0/2-3+1/1-2+1/3(1); ThIII, 0-1+0/2-3+1/1-2+1/3(1); ThIV-V, 0-1+0/2+1/1+1/3(1); ThVI, 0-1+0/1-2+1/1+1/3(1) and ThVII, 0+0/1-2+1/1+1/3(1).

Remarks and discussion. *Siambathynella janineana* sp. nov. is morphologically similar to the type species *S. laorsriae* in generic features (Camacho et al. 2011) such as the number of articles on AI and AII, presence of a male antennal organ on the second article of AI, number of exopod articles on the thoracopod, basipod of male ThVIII with teeth and without setae, the presence of a large endopodal spine on the uropod sympod. The two species are also similar in size, ranging between 1 and 1.8 mm, with females being larger than males in both species. However, both species can be easily distinguished by the antennal organ and the morphology of the lobes on ThVIII in both males and females. The other differences are numerous but more subtle, making them potentially more difficult to discern; however, considered altogether, they reveal two distinct species. In general, these differences relate to relative proportions among articles, specific patterns of setation and relative sizes of setae and spines on AI and AII, mouthparts and other appendages such as the thoracopods, uropod and furca.

The new species (S. janineana) differs from the type species (S. laorsriae) in the following characteristics: 1) the antennal organ of S. janineana is simple, consisting of an expanded area, or protrusion, with one smooth seta; by contrast, S. laorsriae has two "curious structures", one resembling a miniature elephant trunk and the other small, half-ring shaped (see Fig. 2H); 2) articles three and seven of the AI of the new species are larger than the rest of the articles, and the fifth is the smallest, whereas in the type species, the first three and the last two AI articles are similarly sized, as are the fourth and fifth, which are the smallest; 3) differences in setation pattern in terms of setal number and size of some AI articles; also, the new species has two aesthetes on the fifth article, which are absent in the type species; 4) AII is 30% shorter than AI in the new species and 40% shorter in the type species; 5) the new species has fewer teeth on the labrum (12 vs 14 in the type species); 6) the distal claw of the pars molaris of the Md is slightly modified and separated from the rest of claws and expanded in the new species; the type species has the typical parabathynellida morphology usual morphology; 7) the most basal claw of the distal endite of MxI is very thin in the new species; 8) the exopod is longer than the endopod in ThII to ThV, but of equal length in ThVI and ThVII in the new species; in the type species, the exopod and endopod are similar in length in ThII, ThVI and ThVII, but in ThIII to ThV, the exopod is longer than the endopod, and in ThI of both species, the exopod is shorter than the endopod; 9) the fourth article of the endopod in ThII to ThVII is rectangular; it is only square in ThI in the new species and in all Ths of the type



Figure 4. Thoracopod VIII of *Siambathynella janineana* sp. nov. Male holotype except in **C**, **D**. **A** lateroexternal view **B** latero-internal view **C** ThVIII of a male *Siambathynella laorsriae* Camacho, Watiroyram & Brancelj, 2011, latero-internal view **D** ThVIII of the female allotype **E** uropod, dorsal view **F** furcal ramus, dorsal view, and pleotelson. Scale bars in mm.

species; 10) two external setae are present on the second article of the endopod of ThI to ThV, and one seta on the rest of Ths, in the new species; in the type species, only ThI and II present two setae, while the rest only have one seta; 11) the presence of an external seta on the third article of the endopod of all Ths in the new species, which is only present on ThI of the type species; 12) the outer seta of the first article of the exopod is as long as the second article in all Ths of the new species, while it is always shorter than the second article in the type species; 13) the outer seta of the second article of the exopod is long, about half the length of the internal seta in ThII to VII in the new species; it is short, about one-third the length of the internal seta, in the type species; 14) the epipod is smaller in the new species; 15) female ThVIII has two denticles in the new species that are not present in the type species; 16) male ThVIII has a small exopod in the new species, and a large recurved one in the type species; 17) the outer lobe of the male ThVII is large, trapezoidal and well individualized in the new species, while it is reduced and fused to the basipod in the type species; 18) the uropod sympod is five times as long as wide; in S. laorsriae, it is only three times as long as wide; 19) the uropod exopod is 30% longer than the endopod in the new species; the two structures are similar in size in the type species; 20) the strong distal spine of the uropod endopod is 20% longer than the endopod and has with two longitudinal rows of setules in the new species, while in the type species, the spine and endopod are of equal length and the spine has an oblique row of setules; 21) the inner distal setae of the uropod exopod and endopod are half as long as the outer setae in new species; in the type species, they are short, one-third or less the length of the outer setae and 22) the two dorsal plumose setae of the furcal rami differ in length in the new species but are of equal length in the type species.

As previously mentioned, the antennal organ is only present in some Asian species of the genera *Atopobathynella* and *Chilibathynella*, in addition to *Siambathynella*. In *Atopobathynella*, the antennal organ of *A. operculata* and *A. paraoperculata* from India consists of two massive lobes without setae or other structures (Ranga Reddy and Totakura 2015), whereas in *A. nelloreensis*, it is digitiform (Bandari, Shaik and Ranga Reddy 2017). In *Chilibathynella kotumsarensis*, the antennal organ is reduced and represented by two conical, dentate and nearly contiguous hyaline structures (Ranga Reddy 2006).

The two genera of Parabathynellidae known from caves in Thailand are easily distinguishable: species of *Siambathynella* present fewer articles, teeth, claws, setae and spines than those of *Paraeobathynella*. The three species of *Paraeobathynella* have between seven and nine AI articles; six to seven AII articles and a between three and nine exopod articles in all the thoracopods (Camacho et al. 2018). Also, a higher number of teeth, claws and spines on the different appendages is observed in *Paraeobathynella* than in *Siambathynella*.

The discovery of *Siambathynella janineana* sp. nov. adds one more piece to the puzzle of the diversification of genera and species, providing relevant information on the diversity of this group of crustaceans and the biodiversity of groundwater environments.

Ecology. The material of *Siambathynella janineana* sp. nov. studied was collected from a pool located in the upper gallery of Tham Chiang Dao Cave. In addition to these specimens, numerous others were sampled by Janine Gibert either by filtering another pool in the upper gallery using a plankton net with an 0.150 mm mesh [about

305 specimens (sp.) were collected with this method] or by using artificial substrates in the same pool (215 sp.) (Gibert 1987). A few specimens were also found in the terminal sump of the tourist gallery (1 sp.), at the outside emergence (1 sp.) and in a Bou-Rouch pump that was positioned about 25 m downstream of the emergence (40 cm depth and 20 l filtered) (1 sp.). The most abundant populations found in the cave were Bathynellacea, with a total of 523 specimens (Gibert 1987). Other taxa collected included mainly ostracods, a few cyclopoid copepods and rarely gastropods and harpacticoid copepods (Gibert 1987). The temperature of the sampled waters varied between 21 °C and 23 °C during July 1985.

Etymology. The species name "*janineana*" (adjective) is dedicated to Prof. Janine Gibert, who passed away in 2009. She was head of the Groundwater Hydrobiology and Ecology Laboratory (UMR CNRS 5023, Laboratoire d'Ecologie des Hydrosystèmes Fluviaux, Hydrobiologie et Ecologie Souterraines) at Université Claude Bernard Lyon 1 in France. She worked in various international programs on subterranean biodiversity and groundwater ecology from the perspective of environmental sustainability, particularly in the project "Protocols for the ASsessment and Conservation of Aquatic Life In the Subsurface (PASCALIS)". She was also a member of the biospeleological team that led Expedition Thai-Maros in 1985 and was highly active in the sampling of the caves, especially Tham Chiang Dao.

Nomenclatural statement. The life science identifier (LSID) number obtained for the new species is https://zoobank.org/F4366538-281F-40A4-92E5-6BAEE6D3E3E2

Distribution of Thai species of the family Parabathynellidae

Until 2011 (Camacho et al. 2011), the only genus of Parabathynellidae known in Thailand was the monotypic *Siambathynella*. Since then, three species of the genus *Paraeobathynella*, which was previously known only from Vietnam, have also been recorded in Thailand (Camacho et al. 2018). With these four species and the new one of *Siambathynella* described here, which was originally found in 1985, the number of known species of Parabathynellidae in Thailand increases to five. These species have been found in caves in both the northern (Nam Nao National Park, Phetchabun and Chiang Dao District) and southern (Nakhon Si Thammarat Province) regions of the country. Given the many unexplored or unsampled areas in Thailand, and generally in Asia and around the world, undoubtedly many new genera and species await discovery.

The distribution of the two genera present in Thailand, and the six species known in this country and Vietnam, is as follows:

Paraeobathynella Camacho, 2005

P. hanjavanitiana Camacho & Watiroyram, 2018. Type locality: Khao Plu Cave, Khao Ro Subdistrict, Thung Song District, Nakhon Si Thammarat Province, Southern Thailand (coordinates: 09°14'22.00"N, 99°48'72.00"E – Z 56 m a.s.l.). Habitat: cave.

- *P. ratensis* Camacho & Watiroyram, 2018. Type locality: Rat Cave, Kapang Subdistrict, Thung Song District, Nakhon Si Thammarat Province, Southern Thailand (coordinates: 08°02'48.24"N, 99°43'42.48"E – Z 89 m a.s.l.). Habitat: cave.
- *P. siamensis* Camacho & Watiroyram, 2018. Type locality: Khao Krot Cave, Kuanthong Subdistrict, Khanom District, Nakhon Si Thammarat Province, Southern Thailand (coordinates: 08°01'22.50"N, 99°34'36.09"E – Z 45 m a.s.l.). Habitat: cave.
- *P. vietnamensis* Camacho, 2005. Type locality: Hang Trinh Nu, Bo Hon Island, Vinh Ha Long, Vietnam. Habitat: caves. Other localities: Trung Trang and Ang Vem caves, Cat Ba Island, west of Vinh Ha Long, Vietnam.

Siambathynella Camacho, Watiroyram & Brancelj, 2011

- S. janineana sp. nov. Type locality: Tham Chiang Dao Cave, Amphoe Chiang Dao, Chiang Mai Province, Chiang Dao District, Northern Thailand (coordinates: 19°23'38.44"N, 98°55'41".00E – Z 455 m a.s.l.). Habitat: cave.
- S. laorsriae Camacho, Watiroyram & Brancelj, 2011. Type locality: Tham Yai Nam Nao Cave, Nam Nao National Park, Phetchabun, Thailand (coordinates: 16°63'19.0"N, 101°51'57.9"E Z 684 m a.s.l.). Habitat: cave.

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RESEARCH ARTICLE



Two new cavernicolous species of Anillinus Casey (Carabidae, Trechinae, Anillini) from Texas with a revised key to Texas species

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Abstract

Two new species of blind cavernicolous ground beetles in the genus *Anillinus* Casey are described from Texas. Based on the structure of the male genitalia, *Anillinus reddelli* **sp. nov.** (type locality: SW Bypass Cave, Williamson County, Texas) is closely related to *A. forthoodensis* Sokolov and Reddell from the neighboring Bell County. Based on the structure of the male genitalia, *Anillinus bexarensis* **sp. nov.** (type locality: Up the Creek Cave, Bexar County, Texas) is closely related to *A. weismanensis* Sokolov and Kavanaugh from Hays and Bell Counties. Both new species are illustrated with images of the habitus, body parts, and male and female genitalia. Based on morphological data a new grouping of the Texas species is proposed and discussed.

Keywords

cave fauna, distribution, new species, new lineage

Introduction

The genus *Anillinus* Casey is one of the most diverse genera of carabid beetles in the Southern United States. It currently includes more than 60 species distributed across the eastern and central parts of the United States, from Maryland and Indiana in the north, to Florida and Texas in the south (Bousquet 2012; Sokolov et al. 2014; Sokolov

and Schnepp 2021). In the eastern temperate forests (especially in the Appalachian region) the genus is dominated by typical litter-species, however towards the South and to the West, the number of litter-species is drastically decreased while the diversity of subterranean species is increased. In Texas, the westernmost part of the generic range, no litter species are known to occur. At present, the Texas fauna of *Anillinus* comprises seven species, all of which can be treated as subterranean species, and most have been reported from a cave environment (Sokolov et al. 2014).

The last paper focusing on the Anillinus of Texas was published almost a decade ago (Sokolov et al. 2014) and was based on material collected during sampling of cavernicolous taxa in the caves of central Texas. These localities have been intensely monitored because of conservation issues involving troglobitic species threatened by urban development (see U.S. Fish and Wildlife Service, Department of Interior 2000; Paquin and Hedin 2004; Ledford et al. 2012). In that paper, the Texas fauna of Anillinus was reviewed and illustrated, and a key to the species was provided. As a result, this research doubled the known species diversity of the state. However, several female specimens originating from different caves could not be identified at that time, because of the lack of males and the impossibility of examining the male genitalia to determine the taxonomic position of these beetles. A number of superficial characters of the unidentified females suggested that additional new species remained to be discovered in the state. Some time ago, through the courtesy of James R. Reddell (Curator Emeritus of Cave Invertebrates Collection, Biodiversity Center, The University of Texas at Austin, TX), I received new material of Texas Anillini from the caves where the previously unidentified females had been collected. Examination of the material proved the correctness of earlier anticipations, allowing delineation of two new species in the genus. Descriptions of these new species, together with corresponding modifications of a key to the Texas species of Anillinus, serve as the basis for this paper.

Materials and methods

This study is based on the examination of 14 specimens of *Anillinus* collected in the caves of Bexar and Williamson Counties of Texas. Type material of new species is deposited in the collections of the National Museum of Natural History, Washington, DC, USA (**NMNH**), in the Texas Memorial Museum, The University of Texas at Austin, Austin, Texas, USA (**TMM**), and in the collection of the California Academy of Sciences, San Francisco (**CAS**).

Terms used in this paper are of general use, in particular cases for codes of elytral chaetotaxy follow Erwin (1974), for male and female genitalia follow Sokolov and Carlton (2008) and Sokolov et al. (2014).

Extractions and processing of genitalia were made using standard techniques as described by Sokolov and Kavanaugh (2014).

Photographs of the external features of specimens were taken with a Macropod Pro photomacrography system (Macroscopic Solutions, LLC). Digital images of genitalia

All specimens were measured using tpsDig 2.17 (Rohlf 2013) software on digital photographs. Measurements for various body parts are encoded as follows: **ABL** = apparent body length, from clypeus to apex of elytra; **WH** = width of head at level of first orbital setae; **WPm** = maximum width across pronotum; **WPa** = width across anterior angles of pronotum; **WPp** = width across posterior angles of pronotum; **LP** = length of pronotum from base to apex along the midline; **WE** = width of elytra at level of 2^{nd} discal seta; **LE** = length of the elytra, from the apex of the scutellum to the apex of the left elytron. Apparent body length (ABL) measurements are given in mm, others are presented as nine ratios: body parts –WPa/WPp, WPm/WPp, WPm/LP, WE/LE; body proportions – WH/WPm, WPm/WE, LP/LE, LE/ABL, and WE/ABL. All values are given as the mean ± standard deviation.

Results

Order Coleoptera Linnaeus, 1758 Family Carabidae Latreille, 1802 Subfamily Trechinae Bonelli, 1810 Tribe Anillini Jeannel, 1937

Genus Anillinus Casey, 1918

- Anillinus Casey, 1918: 167. Type species: Anillus (Anillinus) carolinae Casey, 1918, by original designation.
- *Micranillodes* Jeannel, 1963a: 57. Synonymy established by Bousquet (2012: 699) and confirmed by Sokolov et al. (2014: 83). Type species: *Micranillodes depressus* Jeannel, 1963a, by original designation.
- *Troglanillus* Jeannel, 1963b: 147. Synonymy established by Barr (1995: 240). Type species: *Troglanillus valentinei* Jeannel, 1963b

Anillinus bexarensis sp. nov.

https://zoobank.org/C17CDC72-318B-491F-9A7E-CF32D3925A26 Figs 1, 2, 5C

Type material. *Holotype*: male, deposited in NMNH, card-mounted, dissected, labeled: \ USA-TX: Bexar Co., Up the Creek Cave, 29.631433°N, 98.559079°W, 12 Mar 2020, J. Owen, UTIC#246598 \ HOLOTYPE *Anillinus bexarensis* Sokolov, 2022 [red label].

Paratypes (8 specimens, deposited in NMNH and TMM). One female, labeled same as holotype, except UTIC#246599 \; 1 male, labeled same as holotype, except , 25 Feb 2020, J. Owen, A. Jensen, UTIC#246585 \; 1 male, labeled same as holotype,

except 5 Mar 2020, J. Owen, UTIC#246593 \, 1 female (pronotum broken, ovipositor sclerites and spermatheca lost), labeled: \TX: Bexar Co., Up the Creek Cave, 14.XI.1995, J. Cokendolpher, J. Reddell, M. Reyes \Texas Memorial Museum Invertebrate Zool Coll #27.141 \; 2 females labeled: \TEXAS: Bexar Co., Constant Sorrow Cave, 29.63554° 98.58514°W, 20 Jan 2020, K. McDermid, L. Pustka, UTIC#246580 \; 1 female labeled: \TEXAS: Bexar Co., Constant Sorrow Cave, 29.63554°N, 31 Jan 2020, K. McDermid, L. Pustka, UTIC#246564 \; 1 female labeled: \TEXAS: Bexar Co., Constant Sorrow Cave, 29.63554°N, 98.58514°W, 31 Jan 2020, K. McDermid, L. Pustka, UTIC#246564 \; 1 female labeled: \TEXAS: Bexar Co., Constant Sorrow Cave, 29.63554°N, 98.58514°W, 19 Mar 2020, J. Owen, UTIC#246623 \.

Additional material. One female (in poor condition, only head, pronotum and abdominal ventrites present, ovipositor sclerites and spermatheca lost), deposited in CAS, labeled: \Zara-3873: TX: Bexar Co., Holy Smoke Cave, 10.XII.2008, P. Sprouse, S. Zappitello \.

Etymology. The specific epithet is a Latinized adjective in the masculine form based on the name of Bexar County, from which the new species is described.

Type locality. U.S.A., Texas, Bexar County, San Antonio, Eisenhower Park area, Up the Creek Cave, 29.631433°N, 98.559079°W.

Recognition. Females of *A. bexarensis* are practically indistinguishable from those of other Texas species of subterranean *Anillinus*. Males of *A. bexarensis* are distinguished from those of the other Texas species by the structure of the median lobe.

Description. Medium-sized for genus (SBL range 1.68-1.88 mm, mean 1.78 ± 0.082 mm, n = 7).

Habitus. Body form (Fig. 1A) subdepressed, subparallel, markedly elongate (WE/SBL 0.37 ± 0.009), head (Fig. 1B) large for genus compared to pronotum (WH/WPm 0.76 ± 0.0207), pronotum wide in comparison to elytra (WPm/WE 0.82 ± 0.020).

Integument. Body rufobrunneous, appendages testaceous. Microsculpture distinct over all dorsal surfaces of head, pronotum and elytra, with isodiametric polygonal meshes.

Head. Labium with mental tooth; mentum and submentum separated by suture.

Prothorax. Pronotum (Fig. 1C) relatively long (LP/LE 0.39 ± 0.017) and transverse (WPm/LP 1.31 ± 0.017), with lateral margins shallowly sinuate and moderately constricted posteriorly (WPm/WPp 1.28 ± 0.026). Anterior angles indistinct, posterior angles nearly rectangular (95–105°). Width between anterior and posterior angles of approximately equal length (WPa/WPp 0.99 ± 0.035). Basal margin slightly concave.

Elytra (Fig. 1A). Slightly and widely depressed along suture, of normal length (LE/SBL 0.59 ± 0.013) and narrow for genus (WE/LE 0.62 ± 0.021), with traces of 5–6 striae. Humeri distinct, rounded, in outline forming obtuse angle with longitudinal axis of body. Lateral margins subparallel, slightly divergent at basal fifth, evenly rounded to apex in apical fourth, without subapical sinuation. Vestiture of elytra short (less than one-third length of discal setae). Apex of elytron truncate with distinct sutural angle.

Legs. First male protarsomere markedly dilated apico-laterally with two rows of adhesive setae ventrally. Male hind legs modified: metafemora triangularly dilated along posteroventral margin with a small tooth at tip of dilation.



Figure 1. Digital images of external features of *Anillinus bexarensis* sp. nov. **A** habitus, dorsal aspect (female, Up the Creek Cave, Bexar County, Texas) **B** head, dorsal aspect (male, Up the Creek Cave, Bexar County, Texas) **C** pronotum, dorsal aspect (male, Up the Creek Cave, Bexar County,Texas). Scale bars: 1.0 mm (**A**); 0.2 mm (**B–C**).



Figure 2. Digital images and ink drawings of male and female genitalia of *Anillinus bexarensis* sp. nov. (Up the Creek Cave, Bexar County, Texas). Male genitalia: **A** median lobe, right lateral aspect; apex to upper left and basal bulb to lower right **B** left paramere, left lateral aspect **C** right paramere, right lateral aspect. Female genitalia: **D** spermatheca **E** ovipositor sclerites. dp–dorsal protuberance, pbp–posterior basal prolongation, ss–spine-like structure. Scale bars: 0.1 mm.

Male genitalia. Median lobe of aedeagus (Fig. 2A) with short basal lobe, long almost rectangularly bent shaft, and with apex enlarged and bent upwards, narrowly rounded at tip. Apical half of shaft with dorsal protuberance only slightly protruded beyond the general contour of the shaft (Fig. 2A, dp). Dorsal margin narrowly sclerotized along almost all its length. Ventral margin enlarged in apical part, with numerous poriferous canals. Dorsal sclerite in the form of a semicircular stylus-like structure, with characteristic posterior basal prolongation (Fig. 2A, pbp). Scaly membranous field occupies almost entire apical third of the shaft, with numerous short spines basally and anteroventrally. Enlarged apical area of median lobe with a dark spine-like structure (Fig. 2a, ss). Left paramere (Fig. 2B) wide, greatly enlarged basally, without long setae. Right paramere (Fig. 2C) long and wide, with numerous (>8) long setae, their length approximately equal to the length of the paramere.

Female genitalia. Ovipositor sclerites (Fig. 2E) typical for *Anillinus*. Gonocoxite 2 unguiform, of moderate length, with slightly curved blade and narrowly rounded apex, with one nematiform and two ensiform setae. Laterotergite with 8–9 setae. Spermatheca with distal part of cornu abruptly dilated. Nodulus short, slightly sclerotized, ramus undifferentiated (Fig. 2D). Spermathecal gland and spermathecal duct shorter than the length of the spermatheca.

Geographical distribution. This species is known only from several caves located in Bexar County, Texas (Fig. 5C).

Way of life. This species has been found only in caves.

Relationships. The presence of a dorsal protuberance on the shaft and the characteristic shape of the dorsal copulative sclerite of the median lobe put *A. bexarensis* together with two other Texan species of *Anillinus*, *A. wisemanensis* Sokolov and Kavanaugh and *A. sinuatus* Jeannel. The general outline of the median lobe and details of the armature of the apical part of the median lobe suggest that *A. bexarensis* is the closest relative of *A. wisemanensis*.

Anillinus reddelli sp. nov.

https://zoobank.org/B4413401-93FB-46C2-AB33-1AB5C0599DA6 Figs 3, 4, 5B

Type material. *Holotype:* male, deposited in NMNH, card-mounted, dissected, labeled: \ USA-TX: Williamson Co., SW Bypass Cave, No 1, TMM # 91,549, Kemble White, 12 Oct 2016 \ HOLOTYPE *Anillinus reddelli* Sokolov des., 2022 [red label].

Paratypes (2 specimens, deposited in NMNH and TMM). One female, dissected, labeled: \ USA-TX: Williamson Co., Beck Horse Cave, TMM # 91,543, Kemble White, 4 Mar 2015 \; 1 male, dissected, labeled: \ TEXAS: Williamson Co., Glenna Mae's Cave, TMM # 91,592, Kemble White, 5 Jun 2015 \.

Additional material. One male (aedeagus lost), deposited in CAS, labeled: \TX: Williamson Co., Lobo's Lair, 13.IX.1991, J. Reddell & M. Reyes \ Texas Memorial Museum Invertebrate Zool Coll #27.142 \; one female, deposited in CAS, labeled: \TX: Williamson Co., Lobo's Lair, 1.IX.1991, W. Elliot, J. Reddell, M. Reyes, M. Warton \ Texas Memorial Museum Invertebrate Zool Coll #27.126 \.



Figure 3. Digital images of external features of *Anillinus reddelli* sp. nov. **A** habitus, dorsal aspect (female, Beck Horse Cave, Williamson County, Texas) **B** head, dorsal aspect (male, SW Bypass Cave, Williamson County, Texas). **C** pronotum, dorsal aspect (male, SW Bypass Cave, Williamson County, Texas). Scale bars: 1.0 mm (**A**); 0.2 mm (**B–C**).



Figure 4. Digital images and ink drawings of male and female genitalia of *Anillinus reddelli* sp. nov. (SW Bypass Cave and Beck Horse Cave, respectively, Williamson County, Texas). Male genitalia: **A** median lobe, right lateral aspect; apex to upper left and basal bulb to lower right **B** left paramere, left lateral aspect **C** right paramere, right lateral aspect. Female genitalia: **D** spermatheca **E** ovipositor sclerites. abp–anterior basal prolongation, ss–spine-like structure. Scale bars: 0.1 mm.

Etymology. The specific epithet is a Latinized eponym in the genitive case and is based on the surname of James R. Reddell, Curator Emeritus of Cave Invertebrates Collection at the University of Texas at Austin, TEXAS, U.S.A., a participant of many speleological expeditions, outstanding explorer of cave fauna, and a collector of a great number of troglobitic invertebrates, including numerous taxa new to science.

Type locality. U.S.A., Texas, Williamson County, SW Bypass Cave.

Recognition. Females of this new species are practically indistinguishable from those of other Texas species of subterranean *Anillinus*. Males of *A. reddelli* are distinguished from those of the other Texas species by the structure of the median lobe.

Description. Medium-sized for genus (SBL range 1.53-1.83 mm, mean 1.67 ± 0.147 mm, n = 3).

Habitus. Body form (Fig. 3A) subdepressed, subparallel, markedly elongate (WE/SBL 0.36 ± 0.004), head (Fig. 3B) large for genus compared to pronotum (WH/WPm 0.77 ± 0.008), pronotum wide in comparison to elytra (WPm/WE 0.87 ± 0.005).

Integument. Body rufotestaceous, appendages testaceous. Microsculpture distinct over all dorsal surfaces of head, pronotum and elytra, with isodiametric polygonal meshes.

Head. Labium with mental tooth; mentum and submentum separated by suture.

Prothorax. Pronotum (Fig. 3A, C) relatively long (LP/LE 0.42 ± 0.014) and transverse (WPm/LP 1.27 ± 0.015), with lateral margins shallowly sinuate and moderately constricted posteriorly (WPm/WPp 1.28 ± 0.027). Anterior angles indistinct, posterior angles nearly rectangular (95–100°). Width between anterior and posterior angles of approximately equal length (WPa/WPp 0.99 ± 0.010). Basal margin slightly concave.

Elytra (Fig. 3A). Slightly and widely depressed along suture, of normal length (LE/SBL 0.58 ± 0.015) and narrow for genus (WE/LE 0.62 ± 0.016), with traces of 5–6 striae. Humeri distinct, rounded, in outline forming obtuse angle with longitudinal axis of body. Lateral margins subparallel, slightly divergent at basal fifth, evenly rounded to apex in apical fourth, without subapical sinuation. Vestiture of elytra short (less than one-third length of discal setae). Apex of elytron of normal shape with distinct sutural angle.

Legs. First male protarsomere markedly dilated apico-laterally, with two rows of adhesive setae ventrally. Male hind legs modified: metafemora triangularly dilated along posteroventral margin with a small tooth at tip of dilation.

Male genitalia. Median lobe of aedeagus (Fig. 4A) with short basal lobe, long rectangularly bent shaft, and with apex enlarged and bent upwards, tapering to angular tip. Dorsal margin strongly sclerotized along almost all its length. Ventral margin enlarged along entire length to basal orifice, with numerous poriferous canals. Dorsal sclerite in form of a semicircular flagellum-like structure, with characteristic anterior basal prolongation (Fig. 4A, abp). Scaly membranous field located between anterior basal prolongation and flagellum of dorsal sclerite. Apical area of shaft with a dark spine-like structure (Fig. 4A, ss). Left paramere (Fig. 4B) wide, greatly enlarged basally, without long setae. Right paramere (Fig. 4C) long and wide with numerous (>8) long setae, their length approximately equal two-thirds of the length of the paramere.



Figure 5. Distribution of the *Anillinus* species by county in central Texas **A** position of the region under question on the map of Texas **B** distribution of *A. reddelli* and its relatives **C** distribution of *A. bexarensis* and its relatives. Non-rectangular heavy black contour on state map shows the genus range in Texas. Stars on insets indicate counties where new species were collected, color-filled counties on insets indicate ranges of the presumed relatives of the appropriate new species.

Female genitalia. Ovipositor sclerites (Fig. 4E) typical for *Anillinus*. Gonocoxite 2 unguiform, of moderate length, with slightly curved blade and acute apex, with one nematiform and two ensiform setae. Laterotergite with 8–9 setae. Spermatheca (Fig. 4D) with distal part of cornu abruptly dilated. Nodulus short, slightly sclerotized, ramus undifferentiated. Spermathecal gland and spermathecal duct shorter than length of the spermatheca.

Geographical distribution. This species is known only from several caves distributed in Williamson County, Texas (Fig. 4B).

Way of life. This species has been found only in caves.

Relationships. The absence of a dorsal protuberance on the shaft, and the characteristic design of the dorsal copulative sclerite of the median lobe place *A. reddelli* in one group with two other Texan species of *Anillinus*, *A. forthoodensis* Sokolov and Reddell and *A. affabilis* (Brues). The general shape of median lobe and details of its apical part suggest that *A. forthoodensis* is the closest relative of *A. reddelli* among the Texan congeners.

Unidentified material

Among 14 specimens examined I was unable to identify one female with the following label data: \Texas: Bexar County, Fobia Cave, 5 March 2017, P. Sprouse \TMM #91,750 \ (not dissected). This female differs significantly from the other specimens of *Anillinus* collected in Bexar County by its smaller size and different body shape. With its proportionally shorter elytra and markedly subparallel habitus, the female closely resembles the specimens of *A. forthoodensis*. The species of *Anillinus* cannot be unequivocally determined by female spermathecae and in our case examination of the male genitalia is needed to clarify the taxonomical status of the population of beetles from Fobia Cave.

The following key to the Texas Anillinus from Sokolov et al. (2014) is modified to accommodate both new species

1	Larger beetles on average (ABL range 1.60–2.00 mm); pronotum with basal margin more or less straight laterally, posterior angles not shifted forward;
	elytral umbilicate series of pores with 8 th and 9 th pores geminate (eo8 and eo9,
	Sokolov et al. 2014, Fig. 2G–I, p. 82) 2
-	Smaller beetles on average (ABL < 1.50 mm); pronotum with basal mar-
	gin oblique laterally, posterior angles shifted forward (Sokolov et al. 2014,
	Fig. 1C, p. 80); elytral umbilicate series of pores with 8 th and 9 th pores disas-
	sociated, 8 th pore situated approximately equidistant from 7 th and 9 th pores
	(Jeannel 1963a, Fig. 17, p. 57)
2	Species with body markedly elongate, subparallel: with pronotum and elytra
	of approximately equal width (WPm/ WE>0.85) and with narrower elytra
	(WE/LE<0.60)
-	Species with body less elongate, more ovoid: with narrower pronotum (WPm/
2	WE<0.85) and wider elytra (WE/LE> 0.60)
3	Apex of elytron widely concave with a long curved spine on the outer margin $f(x) = \frac{1}{2} \frac$
	of incision (Sokolov et al. 2014, Fig. 2J, p. 82); female spermatheca with
	distal part of cornu (dpc) only slightly dilated (Sokolov et al. 2014, Fig. /G,
	p. 92)
_	Apex of erytron without spine faterally, female spermatneca with distal part of corpu markedly dilated (Figs (D))
4	Pronotum markedly elongate (WPm/LP 1 21+0.024), male median lobe
1	with spiny membranous field (Sokolov et al. 2014 Fig. 6N, p. 90)
	A. forthoodensis Sokolov & Reddell
_	Pronotum more transverse (WPm/LP 1.27 ± 0.015); male median lobe with
	scaly membranous field without spines (Fig. 4A)
5	Pronotum with microsculpture distinct at any angle, lateral margins and pos-
	terior angles varied
_	Pronotum with fine microsculpture visible on disc only at certain angles, lat-
	eral margins with shallow basilateral sinuation before the nearly rectangular
	(90–100°) posterior angles (Sokolov et al. 2014, Fig. 1B, p. 80); beetle from
	Bexar County
6	Male with abdominal ventrites modified; median lobe of male aedeagus with-
	out protuberance on dorsal margin (Sokolov et al. 2014, Fig. 6A, p. 90); sper-
	matheca of female with distal part of cornu (dpc) markedly dilated (Sokolov
	et al. 2014, Fig. 7C, p. 92) A. affabilis (Brues)
-	Male with abdominal ventrites simple; spermatheca of female varied7

7 Metafemora of male modified, triangularly dilated medially (Sokolov et al. 2014, Fig. 4E, p. 86); spermatheca of female with distal part of cornu mark-Metafemora of male unmodified, fusiform (Sokolov et al. 2014, Fig. 4G, p.86); median lobe of male with dorsal side evenly rounded, without dorsal protuberance extended beyond the general contour, and with large spinose ventral sclerite (vs) in the inner sac (Sokolov et al. 2014, Fig. 6K, p. 90); spermatheca of female with distal part of cornu only slightly dilated (Sokolov et al. 2014, Fig. 7F, p. 92)..... A. comalensis Sokolov & Kavanaugh 8 Median lobe of males with following characteristics: dorsal protuberance (dp) of shaft extended far beyond the general contour of median lobe, inner sac with scaly membranous field without spines, dorsal copulatory sclerite without posterior basal prolongation (Sokolov et al. 2014, Fig. 6G-J, p. 90). Beetles from Hays and Bell Counties ... A. wisemanensis Sokolov & Kavanaugh Median lobe of males with following characteristics: dorsal protuberance (dp) of shaft less developed, only slightly extended beyond the general contour of median lobe, inner sac with spiny membranous field, dorsal copulatory sclerite with distinctive posterior basal prolongation (pbp) (Fig. 2A). Beetles from

Discussion

With new findings the number of Anillinus species recorded in Texas has reached nine, which surpasses the total number of Anillinus species in the Ouachita and Ozark Mountains of Arkansas (Bousquet 2012; Sokolov et al. 2017), thereby making Texas the richest region of anilline fauna west of the Mississippi River. New morphological data indicate that the first lineage of Anillinus, namely the A. affabilis-forthoodensissinuatus-wisemanensis lineage (Sokolov et al. 2014), is represented by two groups of species distinguished by the shape and internal design of the median lobes in males. The first group encompasses three species, namely A. affabilis, A. forthoodensis, and A. reddelli, characterized by the unmodified apical half of the shaft and the presence of a distinctive anterior prolongation at the basal part of the dorsal copulative sclerite in the inner sac of the median lobe (Fig. 4A, abp). The cumulative range of this group includes three neighboring counties (Fig. 5B). The second group encompasses another three species, namely A. sinuatus, A. weismanensis, and A. bexarensis. Their representatives demonstrate a distinctive protuberance at the apical half of the shaft (Fig. 2A, dp) and a lack of anterior prolongations at the basal part of the dorsal copulative sclerite in the inner sac of the median lobe. The range of this group extends from Bexar County to Bell County (Fig. 5C) and includes the major part of the generic range within the Edwards-Trinity aquifer system (except Coryell County). It is noteworthy that both groups include one species that is recorded in a non-cavernicolous habitat, A. affabilis in the first group and A. sinuatus in the second group, as well as two species collected

only in caves. Morphologically, based on the structure of male genitalia, species that are recorded outside the caves are less derived and, possibly can be treated as the closest relatives of the ancestral forms of the corresponding groups. Supporting evidence for this statement may be the previously described morphological similarity in structure of the male median lobes between *A. sinuatus* and *A. relictus* Sokolov from Alabama (Sokolov 2021), linking the representatives of *Anillinus* occurring to the west of the Mississippi River with their eastern counterparts.

In addition, it makes sense to comment on the unidentified material of Texas *Anillinus* reported in the previous review of Texas species (Sokolov et al. 2014). Among the six unidentified specimens of *Anillinus* mentioned in the paper, two specimens from Lobo's Lair, Williamson County presumably belong to *A. reddelli*, and two females from Bexar County presumably belong to *A. bexarensis*. The status of the other two females labeled: \TX: Coryell Co., Lucky Day Cave, Fort Hood, 27.VI.2009, J. Fant, J. Reddell, M. Reyes \Texas Memorial Museum Invertebrate Zool Coll #70.013 \ and \TX: Bell Co., Sponge Bob Pot, Fort Hood, 17.II.2009, J. Fant, M. Warton \Texas Memorial Museum Invertebrate Zool Coll #69.724 \ remains obscure, and in both cases the examination of males from the corresponding localities is needed. Together with a female from Fobia Cave of Bexar County mentioned above (in the subsection Unidentified material) specimens from Coryell County and from the Sponge Bob Pot, Bell County belong to a cluster of *Anillinus* populations which on further investigation might lead to the recognition of additional new species.

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The mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA; the USDA is an equal opportunity provider and employer.

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RESEARCH ARTICLE



Free living ciliated protists from the chemoautotrophic cave ecosystem of Frasassi (Italy)

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Abstract

This study provides the first report on a community of free-living ciliated protists from the chemoautotrophic cave ecosystem of Frasassi, Italy. This subterranean groundwater ecosystem represents a hotspot of biodiversity that still needs to be fully explored with particular reference to microbial eukaryotes such as protist ciliates. A total of 33 taxa of ciliates were identified along with one species each of flagellate, heliozoans and naked amoebae, from four main sampling sites, namely, Grotta Solfurea (GSO), Lago Verde (LVE), Ramo Solfureo (RSO), and Pozzo dei Cristalli (PDC). The last consists of small microhabitats/ponds presenting different chemical-physical and biological parameters, such as sulfur and nutrient contents and the presence of bacterial biofilms. Furthermore, an analysis of the cryptic ciliate species biosphere as a 'seedbank' of diversity against cave ecosystem disturbance was also performed. This study also highlights some peculiar adaptations of cave-dwelling ciliates not described in their noncave-dwelling conspecifics, such as the extreme photosensitivity of Urocentrum turbo, the cannibalism of Coleps hirtus, the variable number of thorns in Aspidisca species as a defensive response to predation, and the frequent reorganization of ciliary structures in Euplotes aediculatus. The 18S rDNA sequences were generated for five species and were compared with those of the noncave-dwelling conspecifics. Finally, our results shed light on the still largely unknown ciliate diversity in the chemosynthesis-based sulfidic groundwater ecosystem of Frasassi.

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Keywords

Adaptation, cannibalism, cryptic diversity, environmental stress, feeding behaviour, seed bank, sulphur

Introduction

Caves are unique subterranean habitats that have remained relatively stable, except for climatic fluctuations, for thousands of years. They are characterized by complete darkness, nearly constant air and water temperatures, high humidity near saturation, and poor supply of nutrients (Gale 1992). In this regard, cave organisms show a high capacity to thrive and adapt to these peculiar, as well as "extreme", environmental conditions (van Beynen and Townsend 2005; Galassi et al. 2017). Furthermore, these characteristics also suggest that cave organisms might be highly vulnerable to climatic changes and thus may become some of the rarest and most threatened species/biocenoses on Earth (van Beynen and Townsend 2005; Galassi et al. 2017). Survival in these habitats requires specific adaptations for tolerating the stress of living in darkness and other extreme environmental conditions, such as nutrient and energy limitations; relatively low but stable temperatures (within a range from 13.0 to 13.5 °C); toxic levels of gases (e.g., H₂S, CO₂, CH₄); low oxygen concentrations; and variable hydrogen ion concentrations. In this context, the chemoautotrophic cave ecosystem of Frasassi located in the Marche region of central Italy, on the eastern side of the Apennine Mountains, represents one of the most investigated sulfidic cave ecosystems around the world (Galdenzi and Jones 2017). In this hypogenic cave system, Galdenzi et al. (1997) reported the presence of biofilms, signifying that bacterial communities play an important role in hydrogen sulfide oxidation. Moreover, Galdenzi et al. (1999) observed the growth of microbial biofilms, which secrete sulfuric acid (H₂SO₄) with a pH < 1, known as mucolites. Later, Vlasceanu et al. (2000) found that sulfur-oxidizing bacteria on biofilms play an important role in limestone corrosion and are at the base of the cave trophic web.

In addition to bacteria and archaea, several other cave-dwelling organisms, mainly represented by invertebrates, have been identified and described from the Frasassi cave. In the past two decades, independent faunal surveys in the Frasassi cave complex have led to the discovery of several invertebrate species, including some new to science. The list includes mainly Crustacea (Ostracoda, Copepoda, and Amphipoda; Bertolani et al. 1994; Flot et al. 2010; Karaman et al. 2010; Peterson et al. 2013), Mollusca (Gastropoda; Bodon and Cianfanelli 2012), and Platyhelminthes (Stocchino et al. 2017). However, apart from invertebrates, among the different organism types inhabiting cave systems across the world, it is worth mentioning unicellular, eukaryotic ciliated protists. Since the 19th century, cave-dwelling protists have been an interesting, although challenging, topic for researchers. To date, due to the difficulties in sampling in such harsh environments, very few studies have been performed to describe ciliate communities in caves (Gittleson and Hoover 1969, 1970; Sudzuki and Hosoyama 1991; Landolt et al. 1992; Golemansky and Bonnet 1994; Copellotti and Guidolin 1999,

2003; De Luca et al. 2005). Therefore, ciliates have remained largely unexplored, especially those dwelling in karst environments.

Apart from diversity, some morphological, behavioural and physiological adaptations to life in cave systems have also been reported. In particular, Copellotti and Guidolin (1999) found that ciliate species recorded from karst caves in northeastern Italy show unique morphological characteristics (e.g., increase in the number of contractile vacuoles, or body size ratio reduced to almost 30%), and their communities are expected to be more diverse (at least from a morphological point of view) than those of their noncave-dwelling conspecifics as a result of the long-term adaptation to these highly stressful ecosystems. Intraspecific aggressiveness, as an adaptation to maintain populations in resource-depleted environments such as caves or ephemeral habitats, has been reported in several groups, such as arachnids (spiders), crustaceans (hermit crabs), amphibians (toads and salamanders) and reptiles (lizards) (Arnott and Elwood 2008; Cooper et al. 2015; Melotto et al. 2019). However, these morphological and behaviour traits have been scantly studied in the realm of microorganisms such as protists.

The present investigation aims to characterize for the first time the diversity of cave-dwelling ciliated protists from the Frasassi cave ecosystem in the Marche region (Italy; Fig. 1), including the gene sequence (18S rRNA gene) of six species, and to describe some of the morphological and behavioural differences with their noncave-dwelling counterpart ciliate species.

Materials and methods

Sampling locations

Four main sampling locations were selected in the Frasassi cave system (coordinates WGS84-G: 43.402°N, 12.962°E), which are Grotta Solfurea (GSO), Lago Verde (LVE), Ramo Solfureo (RSO) and Pozzo dei Cristalli (PDC) (in Grotta del Fiume Cave) (Fig. 1A, B). The sampling sites were selected on the basis of the presence of sulfidic water pools or springs and were accessed via technical spelunking routes.

The ciliate diversity from site PDC was studied in detail since it is highly diversified and includes seven distinct microhabitats [i.e. Strettoia del Tarlo (SDT), Lago della Scala (LDS), Pozzo dei Cristalli Stream (PCS), Pozzo dei Cristalli Pond (PCP), Hydrogen Sulfide Spring (HSS), Lago Galdenzi (LGA), and Lago della Bottiglia (LDB)], represented by small sulfidic (H_2 S-rich) ponds, streams, and springs as well as deep and shallow muddy, stagnant lakes (Fig. 1B). However, the topological configuration of these sites/microhabitats, and in particular of the highly diversified PDC site, which is located in the close proximity of the Sentino River (which was also sampled with recording of *Urocentrum turbo* noncave-dwelling population), can be dramatically altered following heavy rainfall events that may provoke an increase in water levels inside the cave, resulting in merging all the PDC microhabitats (Fig. 1). For this reason, sites were accessed exclusively during "dry periods" (i.e., far from heavy rainfall events) to collect water/sediment samples in similar ecological conditions across different sampling dates and to make easier access to the different sites. Since the main objective of this study was to perform a study on cave-dwelling protist communities from the Frasassi cave complex, no chemical–physical parameters were measured. Table 1 lists some of the main geochemical parameters (H₂S and O₂ concentrations) recorded by other researchers during the most accessible dry season (Dattagupta et al. 2009; Flot et al. 2010; Bauermeister et al. 2012).

The Italian noncave-dwelling population of *Urocentrum turbo* was isolated in September 2011 from small ponds near the bank of Sentino River, which flows in the deep Frasassi Gorge (43°24'03"N, 12°57'55"E) (Fig. 1A). The Indian noncave-dwelling populations of *Urocentrum turbo* were isolated from water samples collected in July 2021 from a local pond in Khadabandha (21°57'08"N, 85°23'35"E), Odisha, India, and in October 2021 from Khajjiar Lake (32°32'48"N, 76°03'26"E; Ramsar site), Himachal Pradesh, India. The Indian noncave-dwelling population of *Coleps hirtus* was isolated in December 2018 from a natural small pond near the bank of the Ganges River (Hooghly), Kolkata (22°29'26"N, 88°12'28"E). The water samples were transferred to the laboratory at the Zoological Survey of India, Kolkata, for further processing according to Bharti et al. (2019).

Site description	Collection date	[O2] (µM)	[H2S] (µM)
PDC	August 2006	0.2	322
	May 2007	2.5	542
	May-June 2009	12	415
GSO	May 2007	1.2	201
	May-June 2009	51	118
LVE	May 2007	3.6	301
	May-June 2009	2	415
RSO	August 2006	1.0	195
	May 2007	1.6	240
	May-June 2009	10	109

Table 1. Main Geochemical parameters of PDC, GSO, LVE, and RSO (Dattagupta et al. 2009; Flot et al. 2010; Bauermeister et al. 2012).

Ciliate sampling and processing

Periodic sampling was carried out from 2009 to 2011 (Table 2). However, and mainly due to accessibility constraints, it was not always possible to sample all sites during each sampling date since most of these sites were accessible exclusively during the dry season. Water and sediment were collected in half-filled 250 ml sterile plastic bottles and maintained at a temperature close to that of the sampling site by using a 12 V portable refrigerator (GEOSALD SRL, Milan, Italy) until they reached the laboratory at the University of Camerino, Italy (approximately 45 min drive from Frasassi).



Figure 1. A map of the Frasassi cave system showing the location of the main four sampling sites (Map modified after Mariani et al. (2007) **B** simplified map of the PDC showing the seven microhabitats (Drawn by Dr. Alessandro Montanari).

Nytex nets of decreasing mesh size (500 to 150 μ m) were used in succession to filter out crustaceans, sand grains, and other unwanted materials. In the laboratory, all ciliate cell cultures were maintained in a dark chamber at 13 °C. To investigate the possible presence of cryptic ciliate species, each sample was split into two Petri dishes, one containing autoclaved rice/wheat grain to support bacterial growth and the other with the green alga *Chlorogonium elongatum*. These samples were periodically checked for a period of 20 days for the presence of ciliates. Live observations were made using a microscope with bright-field illumination at a magnification of 100–1000×. An Optika microscope camera was employed for photomicrography and video captures. Protargol staining (Kamra and Sapra 1990) and silver nitrate staining (Corliss 1953) were employed to reveal the infraciliature and cortical structures. Classification is according to Lynn (2008).

 Table 2. Sampling sites and calendar for collection of water and sediment samples from 2009 to 2011.

 + indicates sampling performed.

 Sampling sites/dates
 PDC
 LVE
 RSO
 GSO

Sampling sites/dates				PDC				LVE	RSO	GSO
	SDT	РСР	PCS	HSS	LDS	LGA	LDB			
Oct 2009		+	+	+	+	+	+			
Dec 2009					+					
Feb 2010	+									
March 2010	+	+	+	+				+		
April 2010	+									
Sept 2011		+	+	+	+	+	+	+		
Oct 2011									+	+
Total	3	3	3	3	3	2	2	2	1	1

DNA extraction, PCR amplification, cloning and sequencing

DNA extraction was performed according to Thomas et al. (2005). Five to six cells were collected with the help of glass micropipettes and washed three times with autoclaved distilled water. For DNA extraction, 50 μ l of 5% (w/v) Chelex 100 with 2 μ l of Proteinase K (20 mg/ml) solution was added to the sample and then incubated at 37 °C for 30 minutes, followed by incubation at 98 °C for 5 minutes. The reaction mixture was cooled immediately on ice and centrifuged in a microfuge tube for 2–3 seconds at a speed of 16,000 rpm. Without picking the Chelex100 beads, 2 μ l of the supernatant was carefully drawn from the top and stored at 4 °C until PCR amplification. Extracted DNA (2 μ l) was dispensed into a PCR tube containing 5 μ l of distilled water; amplifications by PCR were carried out in a total volume of 50 μ l containing 10× PCR buffer, 3 mM MgCl₂, 0.2 mM of each dNTP, 0.5 mM of each oligonucleotide primer, and 5 U of Taq DNA polymerase (USB). PCR amplification was performed with the universal eukaryotic primers (Medlin et al. 1988) Euk A, 5'-AACCTGGTTGATCCTGCCAGT-3' (forward primer), and Euk B, 5'-TGATC-CTTCTGCAGGTTCACCTAC- 3' (reverse primer). Additionally, the nested primer pairs *Euplotes* 18S (FW 5'-TAG AGG GAC TTT GTG TGC AAC C-3') and *Euplotes* 18S (RV 5'-ATC TCC CTG AAA CAC ACG TTG G-3') were used in combination with universal primers for partial 18S rDNA amplification. The PCR program included initial denaturation at 94 °C for 3 minutes, followed by 35 cycles of 94 °C for 1 minute, 55 °C for 45 seconds, and 72 °C for 80 seconds with a final extension step at 72 °C for 10 minutes. After confirmation of appropriate size by agarose gel electrophoresis, PCR products were purified using the Nucleospin gel extraction kit (Qiagen, Milan, Italy) and inserted into a PGEM-T easy vector system (Promega, Milan, Italy). Plasmids showing the right size inserts (i.e., in a range of 1600–1800 bp) were purified and sequenced at either BMR genomics (Italy) or STAR Seq (Germany).

Results

Ciliate community composition and species richness

In the present study, 36 protist taxa from four different sampling sites within the Frasassi cave system, consisting of 33 ciliate species, one flagellate species, one naked amoeba species and one heliozoan species, were recorded. Flagellates, belonging to the genus *Peranema*, were present in the LDS, HSS and PCP sampling sites, while amoebas were present in the LGA sample, and heliozoans were present in the SDT sampling site.

The 33 identified species of ciliates belong to 8 classes, 15 orders and 25 families (Table 3, Figs 2–6). Two classes contributed more than half of the total species recorded, i.e., class Spirotrichea (11 species belonging to 3 orders and 6 families), with a dominance of 0.35, and class Oligohymenophorea (6 species belonging to 3 orders and 5 families), with a dominance of 0.19 (Tables 3, 4, Figs 2–5). The highest diversity of ciliates was found in PDC (32 species and 24 families), followed by GSO (11 species belonging to 10 orders and 11 families), LVE (5 species belonging to 5 orders and 5 families) and RSO (2 species belonging to 2 orders and 2 families). Peniculida and Sporadotrichida were the only orders common to the four sampling locations, while Colpodida, Haptorida, Heterotrichida, Hymenostomatida and Urostylida were only present in PDC, and Armophorida was only recorded in GSO. Oxytrichidae was the only family present in the four locations (Tables 3, 4).

Ciliate species richness in PDC, considering the complete sampling campaign from 2009–2011 (Table 2), consisted of 32 species, with the maximum number of species recorded at sites PCP (17 species) and SDT (16 species) (Table 3). Site PCP had species that were present in the other sites of PDC; however, some of the species were exclusively reported from site SDT, i.e., *Climacostomum virens, Spirostomum ambiguum, Tachysoma pellionellum, Urostyla* sp. and *Vorticella picta*. The LDS site had the third highest species richness value (13 species), followed by LGA and PCS (11 and 10 species, respectively). The species *Paracolpoda steinii* was exclusively found in LGA, and *Anteholosticha monilata* was found only in LDS. The lowest species richness values were recorded for sites HSS and LDB, with 8 and 6 species, respectively (Table 3).

Table 3. Ciliated Protozoa from the sampling sites of Frasassi caves. Species in bold are the first report for the Italian caves. + indicates presence of the species.

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3. INO.	opecies	Class	Order	ramuy							LVE	Der	Dor
					SDT L	DS H	SS PCS	PCP	, LGA	LDB			
1	Anteholosticha monilata (Kahl, 1932) Berger, 2003	Spirotrichea	Urostylida	Holostichidae		+							
2	Antebolosticha sigmoidea (Foissner, 1982) Berger, 2003	Spirotrichea	Urostylida	Holostichidae	+			+					
\mathcal{C}	Aspidisca turrita (Ehrenberg, 1831) Claparède & Lachmann, 1858	Spirotrichea	Euplotida	Aspidiscidae		+	+	+	+				+
4	Brachonella sp.	Armophorea	Metopida	Metopidae		+		+					+
2	<i>Caenomorpha</i> sp.	Armophorea	Armophorida	Caenomorphidae									+
9	<i>Chilodonella uncinata</i> (Ehrenberg, 1838) Strand, 1928	Phyllopharyngea	Chlamydodontida	Chilodonellidae	+		+	+					+
~	Climacostomum virens (Ehrenberg, 1838) Stein, 1859	Heterotrichea	Heterotrichida	Climacostomidae	+								
8	Coleps hirtus (Müller, 1786) Nitzsch, 1827	Prostomatea	Prorodontida	Colepidae			+	+	+	+	+		+
6	<i>Colpoda inflata</i> (Stokes, 1884) Kahl, 1931	Colpodea	Colpodida	Colpodidae					+	+			
10	Cyrtolophosis mucicola Stokes, 1885	Colpodea	Cyrtolophosidida	Cyrtolophosididae	+	+	+	+	+	+	+		+
11	Dileptus sp.	Litostomatea	Haptorida	Dileptidae			+	+	+				
12	<i>Euplotes aediculatus</i> Pierson, 1943	Spirotrichea	Euplotida	Euplotidae		+							+
13	Euplotes sp.	Spirotrichea	Euplotida	Euplotidae	+	+	+	+	+				
14	<i>Frontonia leucas</i> (Ehrenberg, 1833) Ehrenberg, 1838	Oligohymenophorea	Peniculida	Frontoniidae	+		+						
15	Gonostomum affine (Stein, 1859) Sterki, 1878	Spirotrichea	Sporadotrichida	Gonostomatidae	+	+							
16	Lacrymaria sp.	Litostomatea	Haptorida	Lacrymariidae		+	+	+					
17	Litonotus lamella (Müller, 1773) Foissner et al. 1995	Litostomatea	Pleurostomatida	Litonotidae		+	+	+	+				+
18	Oxytricha setigera Stokes, 1891	Spirotrichea	Sporadotrichida	Oxytrichidae			+	+					
19	Oxytricha sp.	Spirotrichea	Sporadotrichida	Oxytrichidae		+	+	+		+	+	+	+

20 Paracolpoda steinii (Maupas, 1883) Lynn, Colpodea Colpodida Colpodida 21 Paramecium caudatum Ehrenberg, 1833 Oligohymenophorea Peniculida Parameciidae 22 Paramecium caudatum Ehrenberg, 1833 Oligohymenophorea Peniculida Parameciidae 23 Palagothris sp. Prorodontida Urostylidae Urostylidae 24 Spathidium sp. Litostomatea Prorodontida Holophryidae 25 Sprostomum ambiguum (Müller, 1773) Precourichea Heterorrichida Spathididae 25 Sprostomum ambiguum (Müller, 1773) Heterorrichida Sprostomidae Sprostomidae 26 Stentor polymorphus (Müller, 1773) Heterorrichida Sprostomidae Sprostomidae 26 Stentor polymorphus (Müller, 1773) Heterorrichida Sprostomidae Sprostomidae 27 Tadysoma pelitionellum (Müller, 1773) Heterorrichida Sprostomidae Sprostomidae 27 Tadysoma pelitionellum (Müller, 1773) Sprostorichea Heterorrichida Orytrichida 28 Tadysoma pelitionellum (Müller, 1773) Sprostorichea Hymenostomidae Orytrichida	S. No.	Species	Class	Order	Family			PDC			a	VE RS	0 GSO
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Total numbers 8 15 25	33	<i>Vorticellides aquadulcis</i> (Stokes, 1887) Foissner et al. 2010	Oligohymenophorea	Sessilida	Vorticellidae		+	+	+	+		+	+
		Total numbers	8	15	25	16	13 8	8 10	17	11	9	5	11



Figure 2. Percentage of each ciliate order in four locations of the Frasassi cave system to show communities assemblages from 2009 to 2011.



Figure 3. The comparative number of species of each ciliate order in PDC in October 2009 and September 2011.

The distribution of ciliates in PDC was investigated in greater detail for the samples collected in October 2009 and September 2011. In fact, and exclusively on these two sampling dates, it was possible to collect samples from all the investigated microhabitats to eventually observe differences in the community structure (Table 5, Fig. 3). A total of 18 species belonging to 12 orders and 15 families were recorded in PDC in these months. The highest species richness within PDC was found at sites LGA in October 2009 and PCP in September 2011, both with 11 species, although the ciliate communities showed a different species composition on both sampling occasions (Table 5, Figs 3-7). Thus, only three species were present in PCP during both samplings, i.e., Brachonella sp., Oxytricha setigera, and Paruroleptus sp., while no species were detected in the samples taken in September 2011 in LGA. A similar situation occurred in the other sampling sites of PDC, where only a few species from each site were present in both samplings, i.e., Brachonella sp. and Oxytricha sp. in LDS; Chilodonella uncinata, Cyrtolophosis mucicola and Vorticellides aquadulcis in PCS; and Coleps hirtus and Cyrtolophosis mucicola in LDB. The HSS, LDS, PCS, and LDB sites presented 4, 5, 6 and 6 ciliate species, respectively, with only one species, namely, Cyrtolophosis mucicola, common to the four sites (Table 5, Figs 4-7). Despite the differences in the ciliate species composition recorded in October 2009 and September 2011, the ciliate community structure in PDC at the order level remained mostly stable, i.e., from the 12 orders present in PDC on these dates, only two orders were present on one sampling occasion: Colpodida in October 2009 and Hymenostomatida in September 2011 (Fig. 3).

Most of the ciliate species identified in this study can use bacteria as a food resource, which explains why bacterivores, phytobacterivores and nonselective omnivores were the main trophic groups in the Frasassi cave system (Table 4). The presence of other trophic groups was restricted to PDC, and in particular to the sites PCP and LGA. This is likely associated with a greater offer of food sources within these microhabitats, which can host a higher diversity of ciliates than the other sites within PDC, mostly colonized by bacterial feeders. Thus, in PCP and LGA, specialized feeders such as the predator *Dileptus* sp. and *Spathidium* sp. were both found in PCP in October 2009 and in LGA in September 2011. Histophages such as Pelagothrix sp. in LGA were found in September 2011. A special mention should be made of the presence of the nonselective heterotrophic omnivore Coleps hirtus, identified in PCP in October 2009 and in LGA in September 2011. This species is a voracious feeder with a broad food spectrum that in this study in the Frasassi caves also exhibited a cannibalistic behaviour on other conspecific living individuals; never observed, to the best of our knowledge, before in noncave-dwelling counterpart congeners (Tables 4, 5).

Considering PDC as a single sampling site, its ciliate community was composed of 16 species in October 2009 and 14 species in September 2011, with a low species similarity between both samplings, since only a few species were recorded on both occasions, i.e., *Brachonella* sp., *Chilodonella uncinata*, *Coleps hirtus*, *Cyrtolophosis mucicola*, *Oxytricha setigera*, *Oxytricha* sp., *Paruroleptus* sp., and *Vorticellides aquadulcis* (Tables 2–5).



Figure 4. A, B Two specimens of *Litonotus lamella*, arrowheads mark the firm pellicle lines in **A** and the macronucleus in **B**. **C** *Coleps hirtus* **D**, **E** *Gonostomum affine* cells, arrowheads show the curved adoral zone of membranelles typical of the genus **F** *Vorticella picta* **G**, **H** live and protargol stained cells of *Tetrahymena pyriformis*, showing the oral apparatus with three adoral membranelles and paroral membrane (double arrowhead) and two of the ciliary rows (arrowheads) **I**, **J** representative specimens of *Climacostomum virens*, arrowhead in **J** marks the stained macronucleus. **MA** = macronuclear nodule. Scale bars: 25µm.

Cryptic biodiversity

The formation of dormant forms [e.g., resting cysts followed by reversible cytodifferentiation (cryptobiosis)] is a key adaptive strategy frequently adopted by ciliates to persist in harsh ecosystems, such as that represented by the chemoautotrophic cave ecosystem of Frasassi. Cryptic ciliate species biodiversity was also recovered by enriching the freshly collected water samples with autoclaved wheat or rice grains to stimulate bacterial growth and to produce a source of food for the "awakening" of the ciliate cyst dormant stages. Accordingly, while the number of ciliate species was low on freshly collected and observed samples (Table 6), the species richness gradually increased after the enrichment of the cultures. Observation of enriched samples on Days 3, 5 and 7 showed the excystment of dormant forms, thereby increasing the species richness. Cryptic species often recovered on successive days were *Chilodonella uncinata*, *Dileptus* sp., *Litonotus lamella*, *Oxytricha setigera*, *Oxytricha* sp., *Paruroleptus* sp., *Tachysoma pellionellum*, *Tetrahymena pyriformis* and *Vorticellides aquadulcis*.

S. No.	Species	Trophic group Food preferences (Foissner 199 Foissner et al. 1999)		
1	Anteholosticha monilata	Phytobacterivore	B, A, D, C	
2	Anteholosticha sigmoidea	Bacterivore	B, Fungal spores	
3	Aspidisca turrita	Bacterivore	В	
4	Brachonella sp.	Bacterivore	В	
5	Caenomorpha sp.	Bacterivore	B, Sb	
6	Chilodonella uncinata	Phytobacterivore	B, A, D	
7	Climacostomum virens	Non-selective omnivore	B, A, D, Fl, Ta, C, R	
8	Coleps hirtus	Non-selective omnivore	B, A, Fl	
9	Colpoda inflata	Bacterivore	B, Fl	
10	Cyrtolophosis mucicola	Bacterivore	В	
11	Dileptus sp.	Predator	P, M	
12	Euplotes aediculatus	Non-selective omnivore	О	
13	<i>Euplotes</i> sp.	Non-selective omnivore	B, A, D, Fl	
14	Frontonia leucas	Non-selective omnivore	О	
15	Gonostomum affine	Non-selective omnivore	B, Fl, Dt	
16	Lacrymaria sp.	Predator	P, M	
17	Litonotus lamella	Non-selective omnivore	Fl, C	
18	Oxytricha setigera	Bacterivore	B, Fl	
19	<i>Oxytricha</i> sp.	Bacterivore	B, Fl	
20	Paracolpoda steinii	Bacterivore	В	
21	Paramecium caudatum	Phytobacterivore	В, А	
22	Paruroleptus sp.	Non-selective omnivore	О	
23	Pelagothrix sp.	Histophage	Н,	
24	Spathidium sp.	Predator	Р	
25	Spirostomum ambiguum	Phytobacterivore	B, Sb, A, D	
26	Stentor polymorphus	Non-selective omnivore	Fl, C	
27	Tachysoma pellionellum	Strict algivore	B, Cy, A, D	
28	Tetrahymena pyriformis	Bacterivore	В	
29	<i>Trithigmostoma</i> sp.	Strict algivore	A, D	
30	Urocentrum turbo	Phytobacterivore	B, D	
31	<i>Urostyla</i> sp.	Phytobacterivore	B, D	
32	Vorticellides aquadulcis	Bacterivore	В	
33	Vorticella picta	Bacterivore	В	

Table 4. Taxonomic and trophic groups in the Frasassi caves from 2009 to 2011.

A = algae; Am = amoeba; B = bacteria; C = ciliates; Cy = cyanobacteria; D = diatoms; Dt = detritus; Fl = flagellates; H = histophage; M = metazoan; O = omnivore; P = protists; R = rotifers; Sb = sulphur bacteria; Ta = testate amoeba.

Adaptations of some ciliate species in cave environments

Some of the species isolated from the Frasassi caves exhibit some peculiar adaptations that clearly distinguish them from their conspecific, noncave-dwelling, populations. In addition, some species could not be cultured in the laboratory due to their particular and/or demanding culture conditions, e.g., a population of *Urocentrum turbo* isolated from the PDC (microhabitats: SDT, PCS and PCP), LVE and GSO sampling sites was found to be so extremely sensitive to light that even when observed in very low light conditions, the shape of the cells began to distort and eventually burst (Fig. 7B). Conversely, three populations of noncave-dwelling *Urocentrum turbo* isolated from the Sentino River,

Genga, Italy; a high altitude wetland in Himachal Pradesh, India; and a local pond in Odisha, India did not show any sensitivity to light exposure (Fig. 7). These cave- and noncave-dwelling populations also differ with respect to the presence or absence of peculiar extrusive organelles generally known as extrusomes with defensive/offensive functions (e.g., trichocysts, toxicysts), which are absent in the Sentino River population but present in both the Indian and cave populations. Moreover, no differences were found at the level of 18S rRNA gene sequences between the cave-dwelling *U. turbo* populations and their noncave-dwelling populations available in the NCBI database (Table 7).

The peculiar adaptations to the harsh cave ecosystem were observed in the ciliates *Aspidisca turrita, Euplotes aediculatus*, and *Coleps hirtus*. It has been reported that some morphological traits (e.g., furrows, spurs, dorsal ribs, thorns, etc.) may vary in number or size under different growth conditions (Copellotti and Guidolin 1999, 2003). This is the case for *Aspidisca turrita*, which exhibited a variable number of dorsal spines, ranging from one to two or three spines, and *Euplotes aediculatus*, which showed reorganization of the oral apparatus, ventral and dorsal ciliature. In contrast to the "regular" division where two sets of ciliature are formed, the reorganizer shows the formation of a single set of organelles. Our observations show that nearly 20 out of 100 specimens analysed were reorganizers (Fig. 8).

Faced with the impossibility of making resting cysts to survive adverse conditions (starvation, drying, etc.), some species have developed other adaptations, both morphological and behavioural, to survive in harsh environments. An example of these adaptations was observed in the worldwide distributed freshwater ciliate *Coleps hirtus*, a prostomatid ciliate with protective, calcified armour, as well as the ability to inject cytotoxic substances able to immobilize potential prey or predators *via* extrusomes, which hinders the survival of other competitors in the environment (Buonanno et al. 2014). While *Coleps hirtus* is generally known as a histophagous and scavenger feeder, the populations of this species found in the Frasassi caves also exhibited a cannibalistic behaviour on other conspecific living individuals never observed before in noncavedwelling counterpart congeners (Fig. 9 and Suppl. materials 1–4).

SSU rRNA Gene Sequences and characteristics

Through this study, the small subunit rRNA gene sequences of five newly sequenced cave-dwelling ciliate isolates were obtained and blasted in the NCBI database, and the maximum similarity percent with noncave-dwelling species was determined, reported in Table 7, together with the assigned GenBank accession numbers. In particular, the 18S rRNA gene sequence of the cave-dwelling *Vorticella picta* showed the highest identity (98%) with *V. fusca* (DQ190468), whereas that of *Euplotes* sp. had the highest identity (99%) with that of *E. elegans* (DQ309868). Furthermore, the 18S rRNA sequence of the ciliate species identified as *Coleps hirtus hirtus* in a previous study (Buonanno et al. 2014) had 98% similarity with both sequences of *C. viridis* (MT253681) and *C. hirtus* (MT253687). The 18S rRNA gene length and GC content of the six species were as follows: *Coleps hirtus* (1775 bp in length and CG content of 45.1%), *Climacostomum virens* (1711 bp, 47.6%), *Euplotes aediculatus* (1874 bp, 44.3%), *Euplotes* sp. (1600 bp, 42.6%), *Vorticella picta* (1712 bp, 42.2%), and *Urocentrum turbo* (1660 bp, 42.8%).


Figure 5. Photomicrographs of live **A–C** silver impregnated cells **D** from RSO **A** and PDC **B–D** *Paramecium caudatum* **A** and *Frontonia leucas* **B** arrowheads show the cytostomes **C** contracted specimen of *Stentor polymorphus*, arrowhead marks the moniliform macronucleus **D** protargol impregnated specimen of *Anteholosticha monilata* showing the zig-zag cirral row (arrowheads) of fronto-ventral cirri typical of the urostyloid group. **AZM** = adoral zone of membranelles; **MA** = macronuclear nodule. Scale bars: 40μ m.

Discussion

Ciliate community structure: species richness, taxonomic and trophic groups

To date, protist diversity in caves has been scantly investigated, and its knowledge is based on scattered and fragmented published information. However, there have been some reports on the protist diversity from Mexican caves located in the states of San Luis Potosí (Cueva de Los Riscos cave) and Guerrero cave (Osorio-Tafall 1943; Hoffmann et al. 1986; Sigala-Regalado et al. 2011), from Slovenian karst caves (Walochnik and Mulec 2009), and from caves in North America (Holsinger 1966; Barr 1968; Hill et al. 1986; Small et al. 1986). More recently, Baković et al. (2022) investigated the protists of three habitats within the Veternica Cave (Croatia) and reported 47 protist taxa, including 16 ciliates. Regarding Italian caves, Copellotti and Guidolin (1999, 2003) recorded over 100 ciliates from karst caves in Northeast Italy, most of which were new to the Italian checklist (http://www.faunaitalia.it/checklist/). In addition to the classical taxonomic approach, more recently, cave protist diversity has started to be explored by using environmental DNA metabarcoding (eDNA) approaches. A few reports from Appalachian karst caves (USA) and Movile Cave (Romania) were recently published by Reboul et al. (2019) and Cahoon and VanGundy (2022), respectively. Overall, ciliated protists still represent one of the most undersampled and investigated taxa in caves.

The present study shows the presence of a diverse ciliate community in the Frasassi cave system, with 33 ciliate species, (Tables 3, 4, Figs 4–6). The highly diversified site

of PDC, which includes 7 microhabitats, was the most species-rich of the four main sampling sites studied (i.e., PDC, GSO, LVE and RSO), with a total of 32, 11, 5 and 2 ciliate species, respectively (Tables 3, 4, Figs 1-6). The PDC site is composed of a network of different microhabitats, which are connected between themselves exclusively during the wet (rainy) season and mostly separated during the dry season (Fig. 1). Within these microhabitats, the highest species richness recorded at site PCP (17 species) is in contrast with the lower number of ciliate species recorded at site HSS (8 species), which is an extension of site PCP but has a higher sulfur content. It is likely that PCP has a relatively lower sulfur content due to the stream flow, and thus, this progressive dilution of the sulfur concentration along the stream resulted in a richer although less sulfur-tolerant ciliate community. Site SDT, located at the entry point of the cave, had stagnant water with sediments and a bacterial biofilm, providing ideal conditions for the growth of most ciliate species, such as a lack of disturbances related to water flow and an abundant bacterial population as food supply, thus favouring a good richness of ciliate species (n=16). The LDS and LGA sites reached the third and fourth highest numbers of ciliate species (n=13, 11); both sites are characterized by shallow, muddy and stagnant water. The HSS and LDB sites also had low numbers of ciliate species during samplings in October 2009 and September 2011. The HSS is actually a sulfidic water spring with bacterial biofilms, thus allowing the survival of mainly those ciliate species, such as Cyrtolophosis mucicola, Euplotes sp., Frontonia leucas, Lacrymaria sp., Litonotus lamella, and Oxytricha sp., that can possibly tolerate higher sulfur levels (ranging from 322 μ M to 542 μ M; Table 1) and can graze actively on the bacteria and other microorganisms, including ciliates, associated with the biofilm. LDB is a deep pond with a stagnant yet stratified water column (i.e., carbonate vadose water at the surface and sulfidic water at the bottom), which was expected to have a high species number. However, this was not the case, probably due to the presence of Coleps hirtus, a highly active nonselective heterotrophic omnivore, with a high degree of tolerance to stress conditions, such as scarcity of food or extreme pH and temperature variation. Additionally, C. hirtus is equipped with extrusomes, which are known to hinder the survival of many other ciliates once cytotoxic substances are released (Buonanno et al. 2014).

The species composition from the ciliate community in PDC was mostly different in October 2009 and September 2011, with a low species similarity between both samplings and only a few species recorded on both occasions ("resident species"). However, the comparison of taxonomic (at the order level) and trophic groups present in PDC on both dates suggests that the individual species could play similar roles within each taxonomic or trophic group, keeping the ciliate community stable over time.

From the other three main sampling sites, i.e., LVE, RSO and GSO, the lowest number of species was recorded at RSO, with only two species present, *Paramecium caudatum* and *Oxytricha* sp. When compared to the other sites, RSO is the most oligotrophic habitat, characterized by having clear water, sulfur content ranging from 109 μ M to 240 μ M (Table 1), and absence of biofilms. It is likely that the presence of bacterial, flagellate and/ or algal populations adapted to these environments could justify the presence of bacteri-

vores or phytobacterivore ciliates such as *Paramecium* and *Oxytricha*. Similar conditions were reported by Foissner (1997) in a study of the ciliates in four clear water rivers in Germany, where the low abundance of ciliates was attributed to the shortage of nutrients. The samples collected in LVE, a deep stratified lake, presented five ciliate species: *Coleps hirtus, Oxytricha* sp., *Vorticellides aquadulcis, Cyrtolophosis mucicola* and *Urocentrum turbo*. Eleven species were present in GSO samples, namely, *Litonotus lamella, Cyrtolophosis mucicola, Euplotes aediculatus, Brachonella* sp., *Vorticellides aquadulcis* and *Aspidisca turrita* (Table 3, Figs 3–6). The GSO site is represented by a small water pool characterized by a sulfur content ranging from 118 μM to 415 μM (Table 1).



Figure 6. Photomicrographs of live **A**, **E** silver impregnated cells **B–D**, **F**, **G** from GSO **A–D** and PDC **E–G**. **A–D** representative specimens of *Euplotes aediculatus*. Note the contractile vacuole in a live specimen (arrowhead in **A**), the horse shoe shaped macronucleus (arrowhead) **B** and the infraciliature and silverline pattern **C**, **D**. **E–G** *Euplotes* sp. live **E** and ventral and dorsal views of protargol impregnated specimens **F**, **G**. Arrowhead in **E** points to the contractile vacuole. Arrowheads in **F** denote the caudal cirri. Double arrowhead in **G** points to the micronuclei and arrowheads to the dorsal kinety rows. AZM = adoral zone of membranelles; **MA** = macronuclear nodule; **TC** = transverse cirri. Scale bars: 40µm (**A–C**), 20µm (**E–G**).

It was observed that some species were "permanent resident species", i.e., they were recovered from the same site on different sampling campaigns, including *Brachonella* sp., *Chilodonella uncinata*, *Coleps hirtus*, *Cyrtolophosis mucicola*, *Oxytricha setigera*, *Oxytricha* sp., *Paruroleptus* sp., and *Vorticellides aquadulcis*. This indicates that these species might be adapted to that particular habitat with regard to maintaining their population under fluctuating environmental conditions. Furthermore, a curious observation was that the high density of individuals of *Oxytricha* sp. found in PDC covered the whole gastropod shell of *Islamia sulfurea* (Bodon and Cianfanelli 2012), probably feeding on the bacteria involved in their decomposition and depositing resting cysts, which is a strategy to protect themselves from predators as well as excysts when conditions are favourable in a more protected environment. In this regard, a ciliate cyst represents a key food resource for protists as well as invertebrates (Bharti et al. 2020).

Flagellates are more abundant than ciliates and are often mixotrophic, occupying both planktonic and benthic levels (Corliss 2002). The low diversity of flagellates, amoebas, and heliozoans recorded in the present study is because the samples were processed with the main focus on detecting ciliate species. Flagellates and heliozoans found in the water/sediment samples from the Frasassi caves were benthic, and as soon as they appeared in the culture, they grew rapidly, overcoming other protist species.

Furthermore, this study led to the discovery of 14 species that are new to Italian ciliate records, i.e., nearly 40% of the total species identified (Table 3). This indicates that a large gap still exists in published Italian ciliate diversity studies. It is believed that efforts based on an integrative approach (i.e., by molecular, morphological, and behavioural approaches) to study actual ciliate diversity will enrich the Italian list as well as the list of known ciliates worldwide (Bharti et al. 2018; Serra et al. 2020). This is also evident from recent studies where several new species have been documented from Italian soils (Bharti et al. 2014, 2015, 2016, 2017; Kumar et al. 2014).

Cryptic species

Cryptic ciliate species were recovered by enriching samples to encourage the growth of dormant ciliates. The number of ciliate species increased in the enrichment cultures. Previous studies have shown the potential significance of the local 'seedbank' of ciliates. For example, dilution of water from a hypersaline lagoon (Esteban and Finlay 2003) yielded typical freshwater ciliate species, while in a study of a freshwater pond (Fenchel et al. 1997), the number of observed ciliate species increased from 20 to 135 following the use of enrichment cultures. Similarly, the observation of freshly collected cave samples showed few active ciliate species (Tables 5, 6), whereas following enrichment, several other species appeared, mainly those that possess the ability to form resting cysts, such as hypotrich ciliates, while the others were possibly present in one or two specimens that increased their abundance post enrichment. The ability of a

given ciliate species to thrive out of its preferred ecological conditions indicates that if it is dispersed to a different location to which it is not apparently suited, it may adapt and succeed, explaining the global distribution of that species (Finlay 2002). Sometimes, such species fail to produce substantial populations in the new habitat or cannot withstand the environmental or ecological conditions of the new habitat, entering a dormant stage, which results in the so-called 'seedbank' of cryptic species within that habitat (Esteban and Finlay 2010).

S. No.	Species	PDC									
		SDT	LDS	HSS	PCS	РСР	LGA	LDB			
1.	Aspidisca turrita						+				
						+					
2.	Brachonella sp.		+			+					
			+			+					
3.	Chilodonella uncinata				+						
					+	+					
4.	Coleps hirtus						+	+			
					+	+		+			
5.	Colpoda inflata						+	+			
6.	Cyrtolophosis mucicola		+	+	+		+	+			
	Dilat				+	+		+			
/.	Dueptus sp.						+				
0	Eutlates an				+	+					
0.	Euploies sp.						+				
9	Litonotus lamella						+				
			+	+							
10.	Oxytricha setigera					+					
	, ,			+		+					
11.	<i>Oxytricha</i> sp.		+								
			+	+				+			
12.	Paracolpoda steinii						+				
13.	Paruroleptus sp.					+					
						+					
14.	Pelagothrix sp.						+				
15.	Spathidium sp.						+				
						+					
16.	Tachysoma pellionellum										
								+			
17.	Tetrahymena pyriformis										
						+		+			
18.	Vorticellides aquadulcis				+		+				
			+		+	+					
	Total number of species	-	5	4	5	11	11	6			

Table 5. Distribution of ciliate species found in PDC in October 2009 (upper line) and September 2011 (lower line). + indicates presence of the species.

Species		LI	DS			Н	SS			P	CS			PO	CP			LI)B	
Days 1–7	1	3	5	7	1	3	5	7	1	3	5	7	1	3	5	7	1	3	5	7
Aspidisca turrita														+						
Chilodonella uncinata									+		+	+		+	+	+				
Coleps hirtus											+		+	+			+	+	+	+
Cyrtolophosis mucicola		+	+	+	+					+	+	+	+	+		+			+	+
<i>Dileptus</i> sp.												+	+							
Litonotus lamella				+				+												
Oxytricha setigera							+									+				
<i>Oxytricha</i> sp.				+				+										+		+
Paruroleptus sp.																+				
<i>Spathidium</i> sp.														+						
Tachysoma pellionellum																			+	+
Tetrahymena pyriformis														+	+	+		+	+	+
Vorticellides aquadulcis				+							+	+			+					

Table 6. Occurrence of ciliates species after enrichment of the samples collected in September 2011 in PDC with autoclaved rice and the green algae *Chlorogonium elongatum*. + indicates presence of the species.

Adaptations of ciliates species to cave habitats

This study was primarily conducted to obtain an overview of the protist ciliate diversity in the different investigated sites of the Frasassi cave, including possible differences at the behavioural and morphological levels between the cave-dwelling ciliate species and their noncave-dwelling conspecifics. It has been observed that organisms living under sulfidic conditions manifest different morphological, behavioural and physiological adaptations compared to nonsulfidic subsurface animals as a result of different environmental stresses (Engel 2007). In the present study, some of the species isolated from the cave could not be cultured in the laboratory. For example, a cave strain of Urocentrum turbo was found to be extremely photosensitive, and the cells began to burst when they were exposed to light; such an answer likely has an adaptive value as the result of microevolution in perpetually dark habitats (Fig. 7). To date, similar behaviour, although not with such a dramatic response, has been described in a few other ciliated protists, such as Stentor *coeruleus*, which escapes from areas of high light intensity as an antipredation strategy (Cadotte et al. 2007), and the psychrophilic ciliate *Euplotes focardii*, which inhabits the shallow marine coastal sediments of Antarctica (La Terza et al. 2001). In this extremophile ciliate, which shares with *U. turbo* a perpetually dark habitat, exposure to visible light is able to induce the expression of its heat shock protein (hsp) 70 genes as a defensive response (La Terza et al. 2004, 2007; Fulgentini et al. 2015). However, no photosensitivity was recorded in noncave-dwelling populations studied from Italy and India, although the Italian noncave-dwelling population showed an absence of trichocysts. Similar observations were reported by Stoeck et al. (2007) in Urocentrum turbo isolated from a meromictic anoxic alpine lake (Alatsee, Germany), showing high similarity in morphology and gene sequences with described populations but lacking trichocysts, a conspicuous and characteristic feature of this species. The presence of trichocysts in the Frasassi cave ciliate population might be explained considering the extremophile environment and the need to protect themselves against predation, e.g., by other ciliate taxa such as haptorid species

Figure 7. Photomicrographs of live **A**, **B**, **E**, **F** bouin's fixed **C** and protargol-impregnated **D** *Urocentrum turbo* from PDC **A–C** and Sentino River population **D** and Indian populations **E**, **F**. **A** a live specimens of *Urocentrum turbo* **B** specimen about to burst after forming atypical structure as consequence of light exposure **C** specimen fixed with bouin's fixative to avoid bursting (arrowheads mark the macronucleus) and record details **D** a non-cave dwelling population collected outside the cave (Sentino River) of the Frasassi cave, arrowheads marks the macronucleus. Note that the Sentino River specimens were found to be resistant to light exposure though deprived of trichocysts. **E**, **F** the Indian populations were found to be resistant to light exposure and possessed the trichocysts (arrows), double arrowheads mark the caudal cirri. Scale bars: 25 µm.

from the genera *Dileptus, Lacrymaria* and *Spathidium* or the prostomatid *Coleps hirtus,* a nonselective heterotrophic omnivore. In this regard, this is a winning strategy because *U. turbo* is an almost ubiquitous species that has been isolated from all the investigated sites with the only exception of RSO.

It has been demonstrated that some morphological features, such as the dorsal thorn in *Aspidisca turrita* and the dorsal ridges in *A. costata*, are inducible defences formed when the organism is exposed to chemical cues produced by certain predators but are lost or significantly reduced in the absence of these cues (Wicklow 1997). The Frasassi cave strain of *Aspidisca turrita* was found to possess two to three dorsal spines (with respect to its noncave-dwelling conspecific), which could represent a temporary morphological defensive trait induced by the presence of potential predators dwelling inside the cave.

The response of ciliate species to various stress conditions can be extremely variable, i.e., formation of a dormant form (cysts), reduction in size, induction of conjugation, and shedding of the oral apparatus and ciliature, among others. Wan et al. (2019) reported

that the *Stentor coeruleus* shows induction of reorganization of the oral apparatus as well as the ciliature (ventral and dorsal) in response to external stressful stimuli, such as primarily chemical exposures. In this regard, the frequent reorganization events (nearly 20 out 100 specimens analysed) observed in the cave-dwelling population *of Euplotes aediculatus* could be attributed to self-repair mechanisms due to the damage caused by the chemical exposure within the cave (Fig. 8). We suppose that *E. aediculatus* could be sensitive to some of the chemicals (i.e., sulfide) in the cave that are able to cause damage, which in turn triggers the initiation of the formation of a new set of oral apparatus and ciliature. In-depth studies are required to understand the timing and mechanistic aspects of reorganization of ciliature resulting from chemically exposed cells of *E. aediculatus* in the cave.



Figure 8. *Euplotes aediculatus* specimens under different stages of reorganization. Double arrowheads point to the newly formed adoral membranelles and arrows to the fragmented macronucleus. Scale bars: $40 \mu m$.

Furthermore, it was observed that in the raw cultures, C. hirtus increased their number to such an extent that it utilized most of the resources available, thereby hindering the growth of many other ciliates in the same culture medium. The toxicity of Coleps hirtus extrusomes on several ciliates was described by Buonanno et al. (2014), who reported that C. hirtus performs multiple attacks (pack hunting) on its prey, administrating a lethal concentration of toxins that cause its rapid immobilization for easier predation. For example, extrusomes can be used by this ciliate to kill much larger organisms, such as young larvae of zebra fish (Mazanec and Trevarrow 1998). This feeding strategy differs from that of hypotrich ciliates, which capture the food particles by generating a water current using the adoral zone of membranelles, which carries the food through the buccal field until it is engulfed in single or several food vacuoles. *Coleps hirtus* is described as an omnivorous feeder that is attracted by dying or dead organisms, including cells of its own cell type (Falkner 1951; Seravinand Orlovskaja 1977). However, in the present work, we observed a previously unseen cannibalistic behaviour in C. hirtus, consisting of the intraspecific aggression of this species, i.e., it actively hunts its own living conspecific (Fig. 9). The significance of this feeding behaviour for the strains of C. hirtus found in



Figure 9. Photomicrographs showing the details of the cannibalistic behaviour of *Coleps hirtus* **A** a static specimen with straight cilia due to multiple attacks by the predatory cells, arrowheads mark the cilia **B**, **C** same specimens after few seconds with detached cilia (arrowheads) **D**, **E** the predatory cells tries to feed on the dead cell, arrowheads in **D**, **E**, **H–J** points to the dead cell **F** the feeding starts with sucking of the cytoplasm and nuclear apparatus, arrowhead marks the macronuclear nodule **G–J** the predatory cells then feed on the calcified armours plates by breaking them into small pieces. The average length of live specimens of *Coleps hirtus* Frasassi cave population is 50 μm.

the Frasassi caves could be a result of adaptation to these harsh environments, as it provides them with an opportunity to maintain their populations for longer periods, particularly in oligotrophic environments. Although this behaviour has been least studied in microbes, especially single-celled protist ciliates, it is well known in arachnids (spiders), crustaceans (hermit crabs), amphibians (toads and salamanders) and reptiles (lizards), among others, as it has a rather deep impact on populations by affecting their fitness and dynamics (Arnott and Elwood 2008; Cooper et al. 2015; Melotto et al. 2019). *Coleps hirtus* has been widely used as a model organism by ciliate ecologists due to its wide distribution; high range of tolerance to temperature, starvation, and low oxygen concentration; broad food spectrum; etc. (Foissner et al. 1999; Pfister et al. 2002). Our observations are based on several populations of *C. hirtus* isolated from different locations in the Frasassi cave complexes in Italy as well as in small ponds near the bank of the Ganges River (Hooghly), Kolkata, West Bengal, India.

Molecular sequence comparison based on the 18S rRNA gene between cave-dwelling ciliate species and their noncave-dwelling conspecifics showed no appreciable differences, since the similarity values were, in most cases, equal to 99% with the exception of *Coleps hirtus*, which shared a similarity value of 98% with both *C. viridis* and *C. hirtus* (Table 7).

Table 7. Molecular (18S rRNA gene) characterizations of selected cave-dwelling species with non-cavedwelling counterpart.

Species/NCBI Accession number	Sampling site	18S rDNA sequence length (bp)	Highest identity*	Similarity value (%)
Coleps hirtus hirtus (KF177278)	LVE, PDC (PCS, LGA, LGB)	1775	Coleps viridis (MT253681) / Coleps hirtus (MT253687)	98
Climacostomum virens (ON678183)	PDC (SDT)	1711	Climacostomum virens (X65152)	99
Euplotes aediculatus (ON678547)	LVE, PDC (LDS)	1874	Euplotes aediculatus (EU103618)	99
Euplotes sp. (ON678276)	LVE, PDC (SDT, LDS, HSS, LGA)	1600	Euplotes elegans (DQ309868)	99
<i>Vorticella picta</i> (ON678258)	PDC (SDT)	1712	<i>Vorticella fusca</i> (DQ190468)	98
Urocentrum turbo (ON678277)	GSO	1660	Urocentrum turbo (AF255357)	99

* Non-cave-dwelling conspecific.

Regarding the latter point, it is worth mentioning that *Coleps hirtus* has been studied thoroughly recently by Pröschold et al. (2021), which shows that this species possesses high phenotypic plasticity and low genetic variability, thereby suggesting a future revision of the species concept for the genus *Coleps. Coleps hirtus* and *Colep viridis* are mainly separated by the presence of endosymbiont algae in the latter. Pröschold et al. (2021) reported that the presence or absence of endosymbiont algae could possibly be a weak species identification characteristic, at least in the genus *Coleps*, and that the mutual relationship between *Coleps* and algae is facultative. In this regard, the cave population of *Coleps hirtus* did not show the presence of algae, and it could be assumed that this may be due to the absence of light resulting in the absence of symbiosis. Furthermore, the sequence similarity is 98% between the cave and German populations of *Coleps hirtus* described by Pröschold et al. (2021). In this regard, we believe that the genus *Coleps needs* further detailed investigations, based on several populations worldwide, for observations regarding phenotypic plasticity. Thus, we still proceed with the old concept and consider *Coleps hirtus* and *C. viridis* as separate species until further investigation.

Conclusion

Overall, the present study provides a baseline survey of the diversity of cave-dwelling ciliated protists from the different microhabitats of the Frasassi cave ecosystem and describes some peculiar morphological and behavioural differences with their noncave-dwelling conspecifics that have not been substantiated at the molecular level. Thus, these results open the way for further investigations to be conducted *via* integrative taxonomic approaches (i.e., morphology, ontogeny, ecology, behaviour, eDNA, etc.) to better decipher the cryptic diversity of these almost neglected and undersampled taxa of eukaryotic microorganisms and their functional roles within the chemoautotrophic cave ecosystem of Frasassi.

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Supplementary material I

Cannibalistic behaviour of *Coleps hirtus* on other conspecific living individuals (Video 1)

Authors: Daizy Bharti, Santosh Kumar, Federico Buonanno, Claudio Ortenzi, Alessandro Montanari, Pablo Quintela-Alonso, Antonietta La Terza

Data type: Multimedia

- Explanation note: *Coleps hirtus* performing multiple attacks (pack hunting) on its prey, administrating a lethal concentration of toxins that cause its rapid immobilization for easier predation.
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Link: https://doi.org/10.3897/subtbiol.44.96545.suppl1

Supplementary material 2

Cannibalistic behaviour of *Coleps hirtus* on other conspecific living individuals (Video 2)

Authors: Daizy Bharti, Santosh Kumar, Federico Buonanno, Claudio Ortenzi, Alessandro Montanari, Pablo Quintela-Alonso, Antonietta La Terza

Data type: Multimedia

Explanation note: Coleps hirtus feeding on immobilization individual.

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Link: https://doi.org/10.3897/subtbiol.44.96545.suppl2

Supplementary material 3

Cannibalistic behaviour of *Coleps hirtus* on other conspecific living individuals (Video 3)

Authors: Daizy Bharti, Santosh Kumar, Federico Buonanno, Claudio Ortenzi, Alessandro Montanari, Pablo Quintela-Alonso, Antonietta La Terza

Data type: Multimedia

Explanation note: Coleps hirtus feeding on immobilization individual.

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Link: https://doi.org/10.3897/subtbiol.44.96545.suppl3

Supplementary material 4

Cannibalistic behaviour of *Coleps hirtus* on other conspecific living individuals (Video 4)

Authors: Daizy Bharti, Santosh Kumar, Federico Buonanno, Claudio Ortenzi, Alessandro Montanari, Pablo Quintela-Alonso, Antonietta La Terza

Data type: Multimedia

- Explanation note: *Coleps hirtus* feeding on immobilization individual. The cytoplasm has already been fed up and the feeding on armour plates is in process.
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