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Phylogenetic systematics of the enigmatic genus *Horologion* Valentine, 1932 (Coleoptera, Carabidae, Trechinae, Horologionini), with description of a new species from Bath County, Virginia

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Abstract

Horologion Valentine, one of the rarest and most enigmatic carabid beetle genera in the world, was until now known only from the holotype of *Horologion speokoites* Valentine, discovered in 1931 in a small cave in West Virginia. A single specimen of a new species from Virginia was collected in 1991, but overlooked until 2018. DNA sequence data from specimens of this new species, *Horologion hubbardi* **sp. nov.**, collected in 2022 and 2023, as well as a critical examination of the external morphology of both species, allow us to confidently place *Horologion* in the supertribe Trechitae, within a clade containing Bembidarenini and Trechini. A more specific placement as sister to the Gondwanan Bembidarenini is supported by DNA sequence data. Previous hypotheses placing *Horologion* in or near the tribes Anillini, Tachyini, Trechini, Patrobini, and Psydrini are rejected. The existence of two species of *Horologion* on opposite sides of the high mountains of the middle Appalachians suggests that these mountains are where the ancestral *Horologion* populations dispersed from, and predicts the discovery of additional populations and species. All specimens of *H. hubbardi* were collected in or near drip pools, and most were found dead, suggesting that the terrestrial epikarst, rather than caves, is the true habitat of *Horologion*, which explains their extreme rarity since epikarst has not been directly sampled. We recognize the tribe Horologionini, a relict lineage without any close relatives known in the Northern Hemisphere, and an important part of Appalachian biodiversity.

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Keywords
Appalachian Mountains, beetles, DNA, karst, micro-range endemic species, molecular phylogenetics, subterranean biodiversity, troglomorphy

Introduction

For over 80 years, the genus *Horologion* has been known from a single specimen (Fig. 1) collected in a small cave in the Greenbrier Valley of West Virginia (Valentine 1932). This valley is underlaid by the Greenbrier Karst, a massive formation of Mississippian age more than 300 m thick in places, containing over 2000 caves, including some of the longest in the world (Stocks and Shears 2015; White 2018b). These extensive karst features harbor 56 subterranean invertebrate taxa, making the valley a hotspot of cave biodiversity (Culver and Fong 2018; Fong and Culver 2018). *Horologion speokoites* Valentine was discovered on July 12th, 1931 in Arbuckle Cave, a small, shallow cave in a cow pasture (Davies 1965). J. Manson Valentine (1932) found the beetle on a small piece of rotten wood beneath a flowstone formation. The beetle's appearance (small, slow moving) and behavior (when disturbed, it burrowed into the wood) were recognized as unusual, and closer inspection revealed it to be a bizarre carabid that defied classification. Valentine quickly prepared a detailed description, published only eight months after the discovery (Valentine 1932), in which he carefully highlighted the unique combination of morphological characters that prevented him from placing the beetle within any tribe or subfamily of Carabidae. The apparently isolated position of *H. speokoites* and the fact that no one has been able to find it again have earned the species a "near mythical" status (Fong et al. 2007). Although Valentine spent the last half of his life pursuing other research interests such as archaeology, he also maintained an interest in *Horologion* and was still developing ideas on how it could be rediscovered up to his death at the age of 92 (Barr 1994).

The beetle Valentine discovered was small, eyeless and densely pubescent, with a distinctive hourglass shaped body and shorter appendages than most other cave carabids (Fig. 1). Valentine (1932) listed characters the beetle shared with various higher taxa within Carabidae: with Trechini (his "Trechinae"), the beetle shared two supraorbital setae, distinctly impressed frontal grooves, entirely pubescent antennae, mandibular scrobe with a seta, conjunct mesocoxae, and dorsally open aedeagus with pleurisetose parameres; with Bembidiini (his "Bembidiinae") the beetle shared the form of ligula and an asymmetrically bent base of the median lobe of the aedeagus; and with *Patrobus* (placed in his "Pogoninae") the beetle shared similar relative lengths of the ultimate and penultimate palpomeres and a glabrous penultimate palpomere. Valentine concluded that the beetle was most similar to the Trechini, but could not belong there because of the lack of discal setae and recurved apical striae on the elytra, non-dentate male protarsomeres, ligula with reduced number of setae, and an elytral umbilicate series seeming to consist of only six rather than eight punctures. Although his arguments were clearly presented and detailed, they are contradictory in places (e.g., lack of

an aedeagal basal bulb is given as evidence for both *Horologion*+Trechini to the exclusion of Bembidiini and *Horologion*+Bembidiini to the exclusion of Trechini) and some of the characters were either erroneously interpreted (e.g., the procoxae are closed in Trechini, Bembidiini, and *Patrobus*, not open) or have since been demonstrated to be homoplastic (e.g., shape of maxillary palpomeres, reduction of adhesive vestiture on male protarsi, loss of apical recurrent striae) (Maddison et al. 2019).

Perhaps because Valentine's description was so detailed, a redescription or reevaluation of *H. speokoites* has never been published that was based on study of the type itself. Other authors published hypotheses on the placement of the species, but the two most detailed and influential of these explicitly state that they were based solely on the study of Valentine's (1932) description and figures (van Emden 1936; Jeannel 1949). The first came from van Emden (1936), who reached the unlikely conclusion that the genus belonged in the Psydrini. In one of Valentine's drawings (fig. 2), van Emden saw the suggestion of linear transverse impressions on the abdominal ventrites, and it was this character, along with his opinion that four protarsomeres should be considered dilated in *H. speokoites*, that led him to his conclusion. Jeannel (1949) strongly disagreed with van Emden's placement in Psydrini, as well as Valentine's opinion that the genus was most closely related to trechines. He concluded that *Horologion* was most closely related to patrobines, but deserved its own higher taxon. He erected the monotypic family Horologionidae, equivalent with subfamilial, supertribal or tribal rank in modern classifications.

The opinion of van Emden prevailed in Ball's review of the Carabidae of the United States, in which *Horologion* is placed in the tribe Psydrini without further comment (Ball 1960). The key to genera in this work, credited to G. E. Ball and his student R. B. Madge, introduced an unfortunate error: the mesocoxae of *Horologion* were considered disjunct rather than conjunct. Valentine (1932) clearly stated (pp. 3 and 5) and showed (fig. 2) that the mesocoxae are conjunct. This error was repeated by Bell (1967) in his review of coxae in Adephaga, and has persisted in subsequent keys to American carabid genera that have built upon the work of Ball and Madge (Downie and Arnett Jr 1996; Ball and Bousquet 2000).

Barr (1964, 1969, 1971) disagreed with the placement of *Horologion* in Psydrini, and considered that *Horologion* belonged in "Bembidiinae", probably equivalent to what are now considered the tribes Bembidiini, Tachyini, and Anillini, either as a subtribe of Bembidiini or a full tribe. Erwin (1982) hypothesized that *Horologion* belonged within Anillina, based on a similar protibial shape, a placement followed by Sokolov et al. (2004). In the most recent catalog of North American carabids, Bousquet (2012) followed the opinion of Barr, and placed *Horologion* in its own subtribe, Horologionina, of Bembidiini *sensu lato*. Maddison et al. (2019) considered *Horologion* to be *incertae sedis* within Trechitae, but hypothesized a sister relationship with the Lovriciina, a group of four Balkan species in three genera (Giachino et al. 2011). No formal taxonomic changes to the status of Horologionina have been proposed since Bousquet (2012).

In 2018, first author Curt Harden discovered a second specimen of *Horologion* in the Carnegie Museum of Natural History in Pittsburgh, Pennsylvania. The specimen was among recently-mounted material salvaged from evaporated ethanol vials found in

the desk of the late Thomas C. Barr, Jr. after his death in 2011. The beetle had been collected and sent to Barr nearly three decades earlier, in 1991, but Barr apparently did not notice it among the several *Pseudanophthlamus* beetles in the same vial. This *Horologion* specimen possessed impressive humeral carinae ending in curved spines that were unlike any other carabid known from North America (Fig. 2); it was collected in Williams Cave in Bath County, Virginia, on the opposite side of the Appalachian Mountains from Arbuckle Cave. Aside from being dirty and likely unnaturally dark, the specimen was in perfect condition (Suppl. material 3: fig. S1A). However, it was a female, and so a full comparison to Valentine's male specimen of *H. speokoites* could not be made. The specimen was certainly very different from Valentine's and likely represented a new species, but the modified humeri could not be ruled out as an example of sexual dimorphism.

Working from the hypothesis that *Horologion* most likely inhabited deep soils rather than caves (Barr 1969; Culver et al. 1973), considerable effort to collect more specimens from endogean (deep soil) and shallow hypogean habitats (Milieu Souterrain Superficiel or MSS (Mammola et al. 2016)) using buried pitfall traps was exerted in 2018 and 2019 in locations near both *Horologion* caves. Eighty-five buried trap samples were collected in total, and although many interesting discoveries resulted, no specimens of *Horologion* were captured. In 2022 and 2023, several trips into Williams Cave were made, which resulted in the discovery of additional *Horologion* (Fig. 3A–D), including males and fresh material suitable for DNA extraction. Two trips into Arbuckle Cave were unsuccessful.

With several intact specimens of both sexes, DNA sequence data, microhabitat observations, and a re-examination of the type of *H. speokoites*, we find ourselves in a position to offer new insights into the natural history and phylogenetic relationships of this mysterious and long misunderstood genus, and to describe the remarkable new species from Virginia.

Methods

Field collecting

Cave collecting was conducted in entrances, in the "twilight zone" where some light from the entrance still reaches and temperatures are influenced by surface conditions and throughout the extensive dark zone. Cover objects such as dead wood and rocks were turned and surfaces were carefully scanned for activity. Special attention was given to microhabitats with active drip pools. In Williams Cave, several bait stations were set. These consisted of small amounts of various baits (Taleggio and feta cheeses, tuna, peanut butter) placed beneath small piles of stones, and were left in place for 11 days and then inspected visually. Of these methods, visual scanning in and near drip pools was the only one that produced specimens of *Horologion*, which were collected into 95% ethanol using small brushes. Collecting was conducted under permit by the Virginia Department of Conservation and Recreation, Division of Natural Heritage (DCR-DNH).

Morphology

Terminology follows that of Slipinski and Lawrence (2013). For male genitalia, the designations of "dorsal" and "ventral" faces of the median lobe follow typical convention and not their relative positions in repose or when everted. Similarly, designation of the "left" and "right" parameres follows typical convention, and thus is the opposite of Valentine's designations. Terminology of mandibular teeth follows that of Maddison (1993).

The number and position of marginal setiferous punctures commonly called the "umbilicate series" are frequently given taxonomic importance in studies of Trechinae (Jeannel 1941; Tian et al. 2023). The punctures near the apex of the elytra have been subject to various interpretations as to which should be considered part of the umbilicate series (Erwin 1974; Giachino and Vailati 2011; Schmidt et al. 2021). We take a conservative view and interpret the umbilicate series of *Horologion* to consist of eight umbilicate punctures, with a "ninth" puncture being the subapical seta, which appears part of the series due to the lack of a recurrent stria.

Setae on the head were considered fixed if they and their pore-punctures were larger in diameter than the background pubescence, and if they were consistently positioned in the same approximate location across specimens of a species. Similarly, the number of fixed setae on the ligula was determined by counting only the larger and consistently placed setae. Determining setae as fixed does not mean they are necessarily considered homologous across taxa.

Lengths of body sections were made from calibrated images using Adobe Photoshop. Measurements are abbreviated in the description as follows: Apparent Body Length measured from tip of clypeus to apex of elytra (**ABL**), maximum head width (**HW**), maximum pronotum width (**PW**), pronotum posterior width measured at narrowest point (**PbW**), pronotum length along midline (**PL**), maximum elytral width (**EL**), and total antennal length as sum of measured length of each individual antennomere (**AntL**). All measurements were straightforward except for **PbW**, which was complicated by the lack of fixed setae or distinct angles at the posterior of the pronotum that could serve as landmarks. The measurement was taken across the point on each side at which the posterior margin begins to curve upward more steeply. Relative size and shape of some body regions are given as ratios of these measurements.

External structures were examined using Leica M80, M125, and M165 stereoscopes, with and without diffusion, at magnifications from 8× to 120×. Male and female genitalia were dissected from cleared abdomens following DNA extraction or digestion in warm 85% lactic acid, using Dumont #5 forceps (Item nos. 11251-20 and 11252-20, www.finescience.com/) and bent #000 and minuten insect pins held in short pin vises. Genitalia were studied in glycerin on depression slides using a Motic BA300 compound microscope and photographed using a Canon Powershot A2200 digital camera aimed through the eyepiece. Line drawings of genitalia were made by hand and traced using Adobe Illustrator. Digital photographs of

external morphology were taken using a Visionary Digital Passport II system with a Canon 6D SLR and 65-mm MP-E 1–5× macro lens. Focus stacking was performed with Helicon Focus (www.heliconsoft.com). The resulting stacked images contain minor artifacts produced by the stacking process. Scanning electron microscope (SEM) images of uncoated specimens affixed to stubs with double sided tape were taken at 15.0 kV in BSE and BSE3D modes using a Hitachi S-3400 Variable Pressure SEM at the Clemson University Scanning Electron Microscopy Facility in Anderson, SC.

Material examined

Specimens examined are deposited in the following collections: Carnegie Museum of Natural History, Pittsburgh, Pennsylvania (**CMNH**), United States National Museum of Natural History, Washington, D.C. (**USNM**), and Virginia Museum of Natural History, Martinsville, Virginia (**VMNH**).

Taxonomy

We follow the modified biological species concept of Coyne and Orr (2004), and consider species to be discrete, reproductively isolated lineages. We lack DNA data for *H. speokoites*, so our comparison is based largely on morphological structures, with additional evidence from geographic isolation. Our suprageneric framework follows Maddison et al. (2019). Thus, we consider the former subtribes of Bembidiini *sensu lato* to be the separate tribes Bembidiini, Anillini, and Tachyini, and limit the tribe Psydrini to include only *Laccocenus*, *Psydrus*, and *Nomius*.

Molecular phylogenetics

DNA was extracted from the abdomen of the female paratype (voucher CWH-452) with ThermoFisher's GeneJet extraction kit (Vilnius, Lithuania) using the manufacturer's protocol. For the male holotype (voucher CWH-484), the same kit was used but the protocol was modified to extend the incubation period to overnight (~20 hours) and the volume of elution buffer was reduced from 200 µl to 130 µl, in two 65 µl additions incubated for 3 minutes each before centrifuging. Concentration of extracted DNA was quantified using a Qubit 3.0 Fluorometer.

Fragments of two nuclear ribosomal genes (18S and 28S), the mitochondrial protein-coding gene cytochrome oxidase I (*COI*), and five nuclear protein-coding genes (arginine kinase [*ArgK*], carbamoyl phosphate synthetase domain of the rudimentary gene [*CAD2* and *CAD4*], wingless [*Wg*], topoisomerase I [*Topo*], Muscle-Specific Protein 300 [*MSP*]) were amplified using the primers from Folmer et al. (1994), Wild and Maddison (2008), Moulton and Wiegmann (2004), Ward and Downie (2005), Maddison and Cooper (2014), Ober (2002), and Shull et al.

(2001) as listed in Suppl. material 1: table S1, using PCR protocols given in Suppl. material 1: tables S2, S3. For *Horologion*, cleaning and Sanger sequencing of PCR products were performed by Psomagen, Inc. (Maryland, USA). For other taxa, amplified products were cleaned, quantified, and sequenced at the University of Arizona's Genomic and Technology Core Facility using a 3730 XL Applied Biosystems automatic sequencer.

Multiple chromatogram assembly and initial base calls were made using either Geneious (ver. 8.1.8; Auckland, NZ) or with Phred (Green and Ewing 2002) and Phrap (Green 1999) in Mesquite's Chromaseq package (Maddison and Maddison 2023a), with subsequent modifications by Chromaseq and manual inspection.

Sequence alignment was performed in Mesquite (Maddison and Maddison 2023c); 18S and 28S sequences were aligned using the L-INS-I option in MAFFT version 7.490 (Katoh and Standley 2013). Most of the protein-coding genes contained no insertions or deletions, and were aligned manually. Several amino acid insertions and deletions were apparent in *Wg*, *CAD2*, and *MSP*. Sequences of these genes were aligned by first translating the nucleotides to amino acids using Mesquite (Characters>Make New Matrix from>Translate DNA to Protein), then aligning the matrix of amino acids using the same MAFFT settings as for the ribosomal genes, and finally forcing that alignment onto the matrix of nucleotides (Alter>Align DNA to Protein…).

The 28S sequence of *H. hubbardi* contained four large insertions greater than 30 bp in length, the longest one being 136 bp. These and other ambiguously aligned regions of 18S and 28S were excluded using the modified GBLOCKS algorithm in Mesquite (Talavera and Castresana 2007) with the options specified by Maddison et al. (2019): Minimum fraction of identical residues for conserved positions = 0.2, minimum fractions of identical residues for highly-conserved positions = 0.4, counting only fraction within taxa with non-gaps at that position, maximum length of non-conserved blocks = 4, minimum length of a block = 4, fraction of gaps allowed in a character = 0.5, and with sites selected in ambiguously aligned regions.

Our matrix included 259 taxa, representing all tribes of Trechitae as well as members of Patrobini and the three genera of Psydrini *s. str.* (*Psydrus*, *Nomius*, and *Laccocenus*) (Table 1). We included 1,642 sequences from previous studies (Maddison and Ober 2011; Maddison 2012; Maddison and Maruyama 2019; Maddison et al. 2019; Maddison and Porch 2021; LaBonte and Maddison 2023, and references cited therein) and 86 newly acquired sequences with GenBank accession numbers OR500886–OR500913, OR503052–OR503098, OR505843–OR505851, and OR505933–OR505934 (Suppl. material 2: table S4). Among the new sequences, the COI sequence of the male holotype of *H. hubbardi* (OR500887, not included in matrix) and *Topo* sequence of *Tasmanitachoides erwini* (OR503074) are "genseq-1", the sequences from the female paratype of *H. hubbardi* (OR505843, OR505933, OR500886, OR503053, OR503061, OR503098, OR503071, OR503063, OR503052) are "genseq-2", and all other sequences are "genseq-4" (Chakrabarty et al. 2013).

Table 1. List of taxa sampled for molecular phylogenetic analyses. For information on vouchers of taxa other than *Horologion*, including identification resources, see Maddison (2012), Maddison et al. (2019), Maddison and Maruyama (2018) and Maddison and Porch (2021).

Outgroups
Pterostichini
Pterostichus melanarius (Illiger)
Moriomorphini
Amblytelus curtus continentalis Baehr
Mecyclothorax vulcanus (Blackburn)
Melisodera picipennis Westwood
Meonis ater Castelnau
Sitaphe parallelipennis Baehr
Tropopterus canaliculus Liebherr
Psydrini
Laccocenus ambiguus Sloane
Nomius pygmaeus (Dejean)
Psydrus piceus LeConte
Gehringiini
Gehringia olympica Darlington
Trechinae
Supertribe Patrobitae
Lissopogonini
Lissopogonus sp. "Nepal: Likhu Khola"
Patrobini
Diplous aterrimus (Dejean)
Diplous californicus (Motschulsky)
Dimorphopatrobus ludmilae Casale & Sciaky
Parapenetretus sp. "China: Yunnan 1"
Patrobus lecontei Chaudoir
Patrobus longicornis (Say)
Patrobus septentrionis Dejean
Penetretus temporalis Bedel
Platidiolus vandykei Kumakov
Qiangopatrobus sp. "China: Yunnan"
Supertribe Trechitae
Horologionini
Horologion hubbardi sp. nov.
Bembidarenini
Andinodontis muellermotzfeldi Toledano & Erwin
Andinodontis sp. "Ecuador: Vinillos"
Argentinatachoides balli Sallenave, Erwin, & Roig-Juñent
Argentinatachoides setiventre (Nègre)
Argentinatachoides sp. "Argentina: Neuquen"
Bembidarenas reicheellum (Csiki)
Bembidarenas sp. nr. *reicheellum* (Csiki)
Tasmanitachoides angulicollis Baehr
Tasmanitachoides baehri Maddison & Porch
Tasmanitachoides erwini Maddison & Porch
Tasmanitachoides fitzroyi (Darlington)
Tasmanitachoides cf. *gerdi* Baehr
Tasmanitachoides hobarti (Blackburn)
Tasmanitachoides kingi (Darlington)
Tasmanitachoides leai (Sloane)
Tasmanitachoides lutus (Darlington)
Tasmanitachoides murrumbidgensis (Sloane)
Tasmanitachoides rufescens Baehr
Tasmanitachoides wilsoni (Sloane)
Tasmanitachoides sp. "Lerderderg R"
Tasmanitachoides sp. "Angle Crossing #1"
Tasmanitachoides sp. "Angle Crossing #2"
Trechini: Trechodina
Apoplotrechus strigipennis (Fairmaire)
Cnides dostali Donabauer
Cnides sp. "Mexico: Sonora"
Cnides sp. "Ecuador: Orellana"
Cyphotrechodes gibbipennis (Blackburn)
Pachydesus bohemani (Jeannel)
Pachydesus parilis (Péringuey)
Pachydesus parvicollis (Jeannel)
Pachydesus rufipes (Boheman)
Paratrechodes macleayi (Sloane)
Perileptus constricticeps (Sloane)
Perileptus sloanei Moore
Perileptus areolatus (Creutzer)
Sporades sexpunctatus Fauvel
Thalassophilus longicornis (Sturm)
Trechobembix baldiensis baldiensis (Blackburn)
Trechodes alluaudi Jeannel
Trechodes bipartitus (MacLeay)
Trechodes jeanneli jeanneli Mateu
Trechodes sp. "India: Karnataka"
Trechosiella laetula (Péringuey)
Trechini: Trechina
Aepopsis robinii (Laboulbene)
Agonotrechus wuyipeng Deuve
Aphaenops cerberus (Dieck)
Blemus discus (Fabricius)
Bothynotrechus castelnaui (Sloane)
Darlingtonea kentuckensis Valentine
Duvalius boldorii boldorii Jeannel
Epaphiopsis grebennikovi Deuve
Geotrechus orpheus (Dieck)
Homaloderodes germaini Jeannel
Iberotrechus bolivari (Jeannel)
Kenodactylus audouini (Guérin-Méneville)
Mexitrechus cf. *michoacanus* (Bolívar & Pieltain)
Mimotrechus scitulus Moore
Nototrechus unicolor Moore
Omalodera dentimaculata Solier
Omalodera limbata Blanchard
Oxytrechus cf. *lallemandi* Jeannel

Oxytrechus sp. "Chile: Villarrica"
Oxytrechus sp. "Ecuador: Pichan"
Paraphaenops breuilianus (Jeannel)
Paratrechus halffteri Mateu
Paratrechus maddisoni Deuve & Moret
Pseudocnides monolcus (Putzeys)
Pseudocnides rugosifrons (Jeannel)
Tasmanorites intermedius Moore
Trechimorphus cf. *brunneus* Moore
Trechimorphus diemensensis (Bates)
Tropidotrechus bawbawensis Moore
Tropidotrechus victoriae (Blackburn)
Trechinotus flavocinctus Jeannel
Trechisibus antarcticus (Dejean)
Trechisibus cyclopterus group #1
Trechisibus cyclopterus group #2
Trechoblemus westcotti Barr
Trechus arizonae Casey
Trechus coloradensis Schaeffer
Trechus hydropicus beutenmuelleri Jeannel
Trechus humboldti Van Dyke
Trechus obtusus Erichson
Trechus oregonensis Hatch
Xenotrechus denticollis Barr & Krekeler

Anillini

Anillodes sp. "USA: California"
Anillinus erwini Sokolov & Carlton
Anillinus unicoi Sokolov
Anillinus (*langdoni* group) sp. "USA: Georgia"
Argiloborus sp. nr. *imerinae* Jeannel
Binaghites subalpinus (Baudi di Selve)
Caeconannus rotundicollis Jeannel
Geocharidius sp. "Mexico: Chiapas 1"
Geocharidius sp. "Mexico: Chiapas 2"
Illaphanus sp. nr. *matthewsi* Giachino
Medusapyga alsea LaBonte
Medusapyga chehalis LaBonte
Microdipnus jeanneli (Alluaud)
Nesamblyops sp. "New Zealand: Mount Robert"
Nesamblyops sp. "New Zealand: Tirohanga Track"
Serranillus jeanneli Barr
Serranillus dunavani (Jeannel)
Typhlocharis armata Coiffait

Sinozolini

Chaltenia patagonica Roig-Juñent & Cicchino
Phrypeus ricksekeri (Hayward)
Sinozolus sp. "China: Sichuan"

Zolini

Merizodus sp. "Chile: Valdivia"
Oopterus laevicollis Bates
Oopterus laeviventris (Sharp)
Pseudoopterus cf. *latipennis* (Broun)
Sloaneana lamingtonensis Baehr
Sloaneana tasmaniae (Sloane)
Sloaneana sp. "VIC: Acheron Gap"
Zolus wongi Larochelle & Larivière

Bembidiini

Amerizus wingatei (Bland)
Amerizus (*Tiruka*) sp. "China: Yunnan"
Asaphidion alaskanum Wickham
Asaphidion curtum curtum (Heyden)
Asaphidion yukonense Wickham
Bembidion obtusum Audinet-Serville
Bembidion tethys Netolitzky
Bembidion aestuarii (Uéno & Habu)
Bembidion anthracinum Germain
Bembidion approximatum (LeConte)
Bembidion assimile Gyllenhal
Bembidion biguttatum (Fabricius)
Bembidion bimaculatum (Kirby)
Bembidion californicum Hayward
Bembidion clemens Casey
Bembidion ephippium (Marsham)
Bembidion errans Blackburn
Bembidion fortestriatum (Motschulsky)
Bembidion genei illigeri Netolitzky
Bembidion geniculatum Heer
Bembidion incisum Andrewes
Bembidion iridescens (LeConte)
Bembidion kasaharai (Habu)
Bembidion laticeps (LeConte)
Bembidion leptaleum Andrewes
Bembidion levigatum Say
Bembidion lonae Jensen-Haarup
Bembidion lunulatum (Geoffroy)
Bembidion mandibulare Solier
Bembidion mundum (LeConte)
Bembidion nigropiceum (Marsham)
Bembidion quadrimaculatum dubitans (LeConte)
Bembidion planatum (LeConte)
Bembidion planum (Haldeman)
Bembidion punctulatum Drapiez
Bembidion rothfelsi Maddison
Bembidion salinarium Casey
Bembidion solieri Gemminger & Harold
Bembidion turbatum Casey
Bembidion transversale Dejean
Bembidion umbratum (LeConte)
Bembidion variegatum Say
Bembidion versicolor (LeConte)
Bembidion vile (LeConte)
Bembidion wickhami Hayward
Bembidion yokohamae (Bates)
Bembidion (*Hoquedela*) sp. "China: Yunnan"
Bembidion sp. "Inuvik"
Lionepha casta (Casey)
Lionepha disjuncta (Lindroth)
Lionepha pseudoerasa (Lindroth)

Ocys harpaloides (Audinet-Serville)
Orzolina thalassophila Machado
Sinechostictus cribrum stenacrus (De Monte)
Sinechostictus elongatus (Dejean)
Sinechostictus sp. "China: Yunnan"
Pogonini
Diplochaetus planatus (G.H. Horn)
Pogonistes gracilis (Dejean)
Pogonus chalceus (Marsham)
Pogonus meridionalis Dejean
Pogonus texanus Chaudoir
Sirdenus grayii (Wollaston)
Thalassotrechus barbarae (G.H. Horn)
Tachyini: Tachyina
Anomotachys acaroides (Motschulsky)
Elaphropus cf. *haliploides* (Bates) #1
Elaphropus sp. "Madagascar"
Elaphropus sp. "Gabon: Ngounié"
Lymnastis sp. "Australia: Queensland"
Lymnastis sp. "Malaysia: Sabah"
Meotachys riparius Boyd & Erwin
Meotachys sp. "Ecuador: Orellana"
Micratopus sp. "Ecuador: Orellana"
Micratopus sp. "Panama"
Micratopus sp. "USA: Arizona"
Nothoderis rufotestacea (Hayward)
Nothoderis tantilla (Motschulsky)
Nothoderis sp. "Ecuador: Napo 2"
Paratachys bistriatus (Duftschmid)
Paratachys terryli Liebherr
Paratachys vorax (LeConte)
Paratachys sp. "Madagascar"
Paratachys sp. "India: Karnataka"
Paratachys sp. "RSA: Limpopo"
Pericompsus braziliensis (Sahlberg)
Pericompsus australis (Schaum)
Pericompsus circuliformis (Solier)
Pericompsus laetulus LeConte
Pericompsus metallicus Bates
Pericompsus punctipennis (Macleay)
Pericompsus sellatus LeConte
Pericompsus semistriatus (Blackburn)
Polyderis laeva (Say)
Polyderis ochrioides (Darlington)
Porotachys bisulcatus (Nicolai)
Porotachys ottomanus Schweiger
Tachys argentinicus Csiki
Tachys corax LeConte
Tachys luxus Andrewes
Tachys scutellaris Stephens
Tachys vittiger LeConte
Tachyta (*Eurytachyta*) sp. "Malaysia: Sarawak"
Tachyta (*Paratachyta*) sp. nr. *philipi* #2
Tachyta inornata (Say)
Tachyta picina (Boheman)
Tachyura apicalis (Boheman)
Tachyura loriae (Andrewes)
Tachyura nervosa (Sloane)
Tachyura sp. nr. *obesula* (LeConte)
Tachyura sp. "RSA: North Cape"
Tachyini: Xystosomina
Erwiniana eugeneae (Erwin)
Erwiniana hilaris (Bates)
Erwiniana sp. nr. *crassa* (Erwin)
Gouleta cayennense (Dejean)
Kiwitachys antarcticus (Bates)
Kiwitachys latipennis (Sharp)
Mioptachys sp. nr. *oopteroides* Bates
Mioptachys sp. "Ecuador: Bellavista"
Mioptachys flavicauda (Say)
Mioptachys sp. "Peru: Iquitos"
Philipis bicolor Baehr
Philipis lawrencei Baehr
Philipis subtropica Baehr

Maximum likelihood analyses of single gene and concatenated matrices were conducted using IQ-TREE version 2.2.0 (Minh et al. 2020) through Mesquite's Zephyr package (Maddison and Maddison 2023b), with 100 searches performed for single genes and 200 searches performed for the concatenated matrix. ModelFinder (Kalyaanamoorthy et al. 2017) was used to find the optimal model of evolution. Single gene analyses of 28S and 18S were unpartitioned and the MFP option was chosen. For the single gene analyses of the protein coding genes, each of the three codon positions was treated as a part and the TESTMERGE option was used to select the best partition scheme and model for each. The TESTMERGE option was also used for the concatenated matrix, starting with 23 parts (one for each of the ribosomal genes and one for each codon position of each protein coding gene, with the two noncontiguous sections of CAD treated separately).

Clade support was measured with standard nonparametric bootstrapping using IQ-TREE, with 500 bootstrap replicates for single genes and the 8-gene concatenated matrix. Ultrafast bootstrapping was also performed for each of these matrices in IQ-TREE, with 1000 replicates, including the SH-aLRT test with 1000 replicates (Hoang et al. 2018). Standard bootstrap support for and against clades was assessed using the "Clade Frequencies in Trees" feature and the standard bootstrap trees in Mesquite. Ultrafast bootstrap support for and against clades was assessed by visually examining the ultrafast bootstrap trees.

A NEXUS file containing the data matrices and the inferred trees has been deposited in Dryad (available at doi:10.5061/dryad.73n5tb33p).

Results

Molecular phylogenetics

DNA was successfully extracted from both fresh *Horologion* specimens. The extraction from the female paratype had low concentration of DNA (0.0380 ng/μL). Nevertheless, sequences of all 8 target genes were successfully amplified. The extraction from the male holotype had a much higher concentration of DNA (0.220 ng/μL).

Maximum likelihood analysis of the 8-gene concatenated matrix produced a tree with a topology of deeper lineages nearly identical to that of Maddison et al. (2019), except that the tribe Sinozolini is recovered as sister to the remaining tribes of their "Clade B2" (Fig. 4). Trechitae including *Horologion* is strongly supported by the 8-gene matrix (standard bootstrap support value [SBS] of 100%) and moderately supported by the single genes 28S (SBS 80%) and *Wg* (SBS 58%), with weaker support from *CAD4* (SBS 25%) and *MSP* (SBS 24%) (Fig. 5). A clade consisting of *Horologion*, Bembidarenini and Trechini is strongly supported by the 8-gene matrix (SBS 90%), and weakly supported by 28S (SBS 48%) and *CAD4* (SBS 23%). *Horologion* is recovered as sister to the tribe Bembidarenini in the 8-gene, 18S, 28S, *Wg*, and *Topo* trees. This *Horologion* + Bembidarenini clade is moderately supported in the 8-gene analysis (SBS 72%) and weakly supported in 18S (SBS 6%), 28S (SBS 15%), *Wg* (SBS 26%), and *Topo* (SBS 20%) trees. *CAD2* and *CAD4* gene trees also recovered *Horologion* in a clade including Bembidarenini and Trechini, but not as sister to Bembidarenini and not with strong support; in *CAD2*, *Gehringia* is also within this clade (SBS 7%). The remaining three single gene trees differ in their placement of *Horologion*, all with low support: *MSP* recovered *Horologion* as sister to the remaining Trechitae (with SBS of 6% for Trechitae excluding *Horologion*), *ArgK* recovered *Horologion* as sister to Patrobini (SBS 17%, Fig. 6), and *COI* placed *Horologion* within a clade including members of Bembidarenini, Tachyini, Trechini, and the genus *Gehringia* (SBS 2%). Suppl. material 4: figs S5–S14 show the ML, SBS and ultrafast bootstrap consensus trees from all analyses.

Taxonomy

Horologionini Jeannel, 1949

Horologionidae Jeannel, 1949: 91. Type genus: *Horologion* Valentine, 1932.
Horologionini: Barr (1964: 1).
Horologionina: Bousquet (2012: 711); Ortuño and Arillo (2015: 584), Maddison et al. (2019: 171).

Diagnosis. The tribe consists of a single genus, *Horologion*, described in detail below. Members of the tribe possess characteristics of the supertribe Trechitae and several character states that, in combination, distinguish them from other tribes of Carabidae: eyes absent, frontal furrows weakly impressed, penultimate maxillary palpomeres glabrous, apical maxillary palpomeres elongate and narrow, pronotal lateral margins and hind angles absent, elytral humeri with carinate shelf, elytron with a single discal setigerous puncture, apical recurrent stria absent, mesoventrite bell-shaped and extended anteriorly, mesocoxae and metacoxae separated by processes of mesoventrite and metaventrite, all surfaces of external integument except for mouthparts and abdominal tergites generally setose, median lobe of aedeagus open dorsally, spermatheca with attached gland, female genital segment subtriangular, gonocoxites slender and largely glabrous.

Horologion Valentine, 1932

Description. Small, eyeless, pubescent beetles with short appendages and pedunculate body form. Head with one supraorbital seta on each side and a posterior row of three to five pairs of inwardly curved macrosetae. Mandibles with scrobal seta present, elongate and similar in shape. Clypeus with two pairs of fixed setae. Head with frontal furrows weakly impressed and poorly defined. Labrum with six fixed setae. Ligula with four large fixed setae, inner pair fused. Head ventrally with suborbital seta on each side. Pronotum strongly constricted posteriorly, lacking posterior angles and posterior marginal setae. Elytral humeri modified, with a raised carinate shelf of variable length and shape. Eight umbilicate punctures along margin of elytra, subapical seta (sensu Schmidt et al. 2021) present, not separated from umbilicate series by carinate interval. Apical recurrent stria absent. One discal seta in third elytral interval. Elytral striae poorly defined, shallow and coarsely punctate. Preapical plica present, well developed. Tarsomeres of all legs short and transverse in both sexes. Males with two basal protarsomeres each bearing a row of adhesive setae ventrally, either both markedly expanded and dentate on inner margin (*H. hubbardi*) or with only the first protarsomere moderately expanded and weakly dentate on inner margin (*H. speokoites*). Procoxae closed posteriorly. Mesocoxae conjunct. Mesoventrite elongate and bell-shaped, coarsely sculptured, with median depression flanked by coarsely setose carinae. Metacoxae widely separated. Median lobe of aedeagus open dorsally,

membranous along entire dorsal margin and most of ventral face. Internal sac with flagellum present, swollen basally, broadly curved and rotated dorsally so that it appears as a complex sclerotized structure in lateral aspect. Spermathecal duct broad, with abrupt U-shaped bend proximally. Spermatheca small and saclike. Spermathecal gland present and elongate. Gonocoxites slender and glabrous except for a single small seta on inner margin near apex. Tergite of female genital segment strongly sclerotized and forming a V-shaped bridge behind gonocoxites.

Horologion speokoites **Valentine, 1932**

Figs 1, 7, 8C, 9A–C, 11A; Suppl. material 3: fig. S2A, B

Material examined. ***Holotype*** **male** (USNM), glued to clear plastic point with bit of wax on pin below point. Right metatibia and metatarsus missing. Right antenna (except for scape and pedicel) and right protibia (except for base) and protarsus removed. Two plastic cards with blocks of Canada balsam pinned beneath specimen, the top one containing genitalia and the bottom one containing the right antenna, protibia and protarsus. Original labels (Fig. 7): "Arbuckle's Cave Maxwelton, W.Va. July 12 1931 J.M. Valentine" "On bit of wet decaying wood, muddy floor of lower level" "♂ Type gen. Type sp. [red paper]" "U.S.N.M. TYPE 44255 [red paper]" "23" "Horologion speokoites Valentine detVal. 1932 HOLOTYPE [white label with red line drawn on each side]" "USNMENT [QR Code] 01374911".

Diagnosis. The lone specimen of this species differs from *H. hubbardi* in the following characters: the carinal shelf of the humerus is shorter and does not end in a prominent curved spine; the elytral disc is more convex, though not nearly as convex as in Valentine's (1932) illustration (his fig. 2) of the lateral aspect (Suppl. material 3: fig. S2B); the two basal protarsomeres are not as strongly expanded and only the first is dentate on the inner margin, slightly so (Fig. 8C); the parameres are broader and bear more than three apical setae (Fig. 9B); the median lobe is more strongly curved ventrally (Fig. 9A) and is slightly bent asymmetrically in dorsal aspect (Fig. 9C); and the flagellum has a distinct sinuation (Fig. 9C).

Notes. Our examination of the type of *H. speokoites* revealed that the specimen differs from Valentine's description and drawings in several important characters. The first protarsomere is asymmetrical and distinctly dentate on the inner margin, and the second is slightly asymmetrical though not dentate (Fig. 8C). The third protarsomere, which Valentine considered expanded, is the same shape as in female specimens of *H. hubbardi* (Fig. 8B), and should therefore not be considered modified. The right elytron bears a prominent discal seta in the third interval. At 100×, using a Leica M125 stereoscope with strong diffuse light directed from the posterior of the specimen, both the pore at the base of the discal seta on the right elytron and the pore (without seta) on the left elytron were clearly visible, and both were located just posterior to the level of the fourth umbilicate puncture. The carinae that Valentine illustrated extending from the humeri onto the disc of the elytra do not exist; the disc in this region is smooth,

Figure 1. *Horologion speokoites* holotype (USNM), dorsal habitus. Scale bar: 1 mm.

and the humeral carinae end well before the level of the parascutellar setae. The plastic on which the genitalia are mounted in balsam is roughly textured, and the structures are heavily cleared. We were unable to study them in right lateral or dorsal aspects. The ventral margin of the median lobe appears to not be as strongly curved as Valentine's drawing suggests, but due to the condition of the preparation and the fact that the parameres are still attached, we are unable to discern the true shape of the median lobe.

Our examination of the specimen also confirms Valentine's (1932) original report of the collection date; in the literature, the year of the collection has been variously reported as "1931" (Valentine 1932), "1930" (Barr 1994) and "1938" (Culver et al. 2012).

***Horologion hubbardi* Harden & Davidson, sp. nov.**
https://zoobank.org/F34C3E90-0136-4D25-A5B0-3E1E384BAF12
Figs 2, 3D, 8, 9D–H, 10, 11B–E, 12; Suppl. material 3: figs S1, S3, S4A

Type material. ***Holotype* male** (Suppl. material 3: fig. S1B) (CMNH), point mounted, abdominal ventrites and right protibia and protarsus glued to point, genitalia in plastic glycerin microvial pinned beneath specimen labels. Original labels: "USA: VIRGINIA, Bath Co. Williams Cave. 29.March.2023. T. Malabad, C. Harden, K. Kosič Ficco. Found floating on pool surface." "Harden DNA Voucher CWH-484 *H.* m Ext. 12/April/2023 [green-bordered cardstock]" "[QR code] CMNH-IZ 769,132" "HOLOTYPE *Horologion hubbardi* ♂ Harden & Davidson [computer printed on red cardstock]". *COI* GenBank accession: OR500887.

***Paratypes* (n = 4):** One female (VMNH), point mounted, abdominal ventrites and genitalia in glass glycerin vial pinned beneath specimen, labeled "USA: VIRGINIA, Bath Co. Williams Cave. 2.August.2022. T. Malabad, D. Hubbard, C. Harden. Active on ground near drip pool." "Harden DNA Voucher CWH-452 H. Williams F Ext. 7/August/2022" [green-bordered cardstock]. GenBank: OR505843, OR505933, OR500886, OR503053, OR503061, OR503098, OR503071, OR503063, OR503052.

One female (CMNH), point mounted, not dissected, labeled "VIRGINIA: Bath County, Williams' Cave, Sep 8 1991" "D.A. Hubbard" "THOMAS C. BARR COLLECTION 2011 Acc. No. 38.014" "VANHP # : Hubbard VA: Co: Bath Loc: Williams Cave Date: 8 Sept 91." "[QR code] CMNH-IZ 769,133".

Two males (VMNH), dry mounted with genitalia in glycerin, missing most of antennae and legs, labeled "USA: VIRGINIA, Bath Co. Williams Cave. 28.March.2023. T Malabad, K. Kosič Ficco, CW Harden. Found dead.".

Other material. Fragments of three specimens, one male, one female, and one unknown sex, in alcohol vials (VMNH): Found dead in or near small pools, Williams Cave, 29 March 2023.

Diagnosis. From *Horologion speokoites*, this species differs in the following external characters: the elytral humeri have longer carinal shelves that terminate in a sharp, curved spine (Fig. 10D); the elytra are flatter (Fig. 11); and the two basal protarsomeres of males are asymmetrically dilated and spinose on their inner margin (Fig. 8A). The male genitalia (Fig. 9D–G) also differ from those of *H. speokoites* (Fig. 9A–C): the parameres are smaller and each bear three apical setae, the median lobe is straighter ventrally, not twisted from plane of basal lobes, with a smaller and more symmetrical apex, and the flagellum of the internal sac is slightly longer and without a distinct sinuation.

Description. ***Habitus*:** Average sized for Trechinae (**ABL** = 3.16–3.20 mm), pubescent, without trace of eyes. Variable in color, dark castaneous in the 1991 specimen

Figure 2. *Horologion hubbardi* female paratype, voucher CWH-452 (VMNH), dorsal habitus. Abdomen removed for DNA extraction. Scale bar: 1 mm.

(Suppl. material 3: fig. S1A) (possibly stained due to unusual ethanol preservation) and lighter in fresher specimens (Fig. 2, Suppl. material 3: fig. S1B); integument strongly sclerotized; proportions delicate, with pedunculate pro-mesothoracic junction; elytra vase shaped, with prominent humeral carinae ending in curved spines. Appendages relatively short; body flattened dorsoventrally.

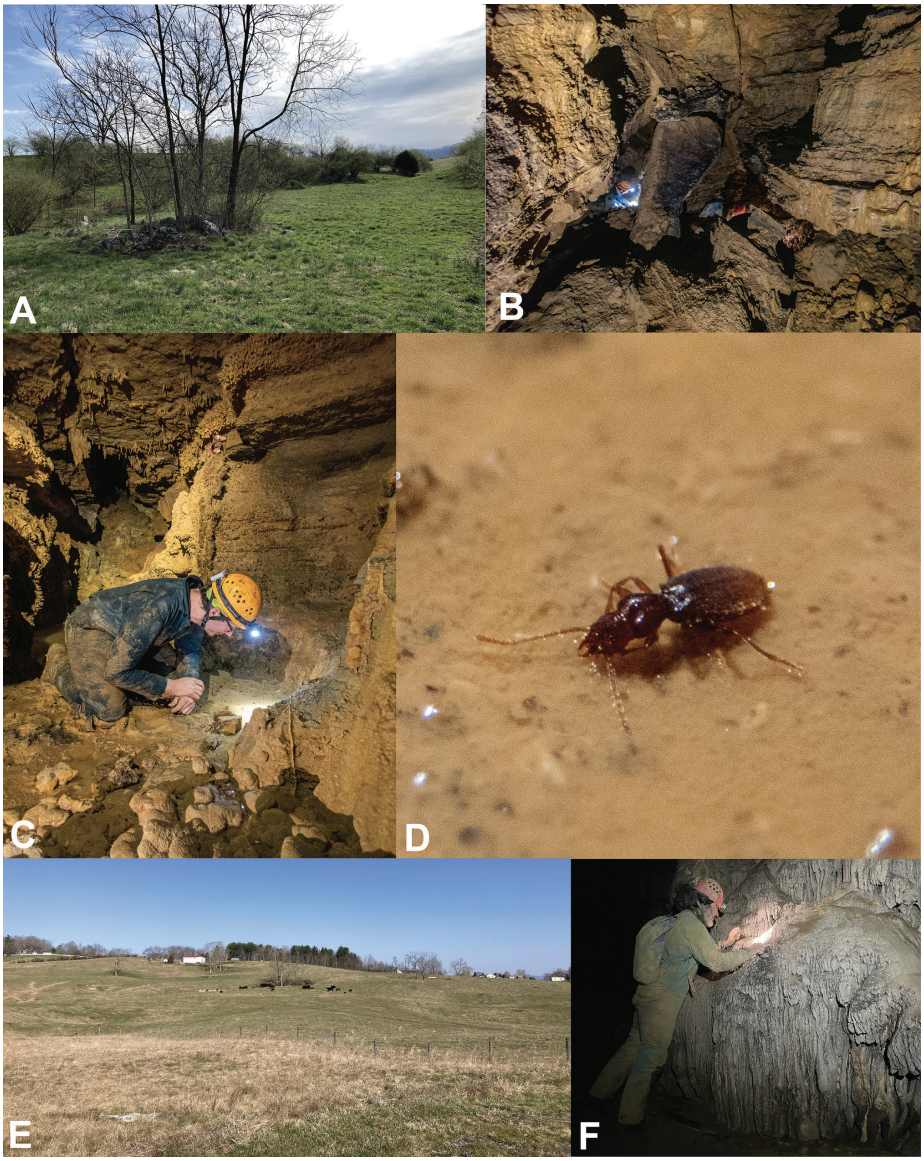

Figure 3. Habitat of species of *Horologion* **A** surface habitat at Williams Cave, Bath Co., VA **B** C. Harden (yellow helmet) and T. Malabad (red helmet) in microhabitat of *H. hubbardi* voucher CWH-452 in Williams Cave **C** C. Harden examining live *H. hubbardi* holotype in Williams Cave **D** live holotype male of *H. hubbardi* on surface of drip pool in Williams Cave **E** surface habitat at Arbuckle Cave, Greenbrier Co., WV **F** T. Malabad examining flowstone in Arbuckle Cave. Photo credit: C. Harden (**A, E**), K. Kosič Ficco (**B–D**), M. Miller (**F**).

***Head*:** Relatively large (**HW/PW** = 0.84–0.86); temples rounded; eyes entirely absent. Dorsal surface evenly covered with short, light-colored setae set in coarse circular pits. Microsculpture consisting of weakly impressed, irregular scalelike sculpticells,

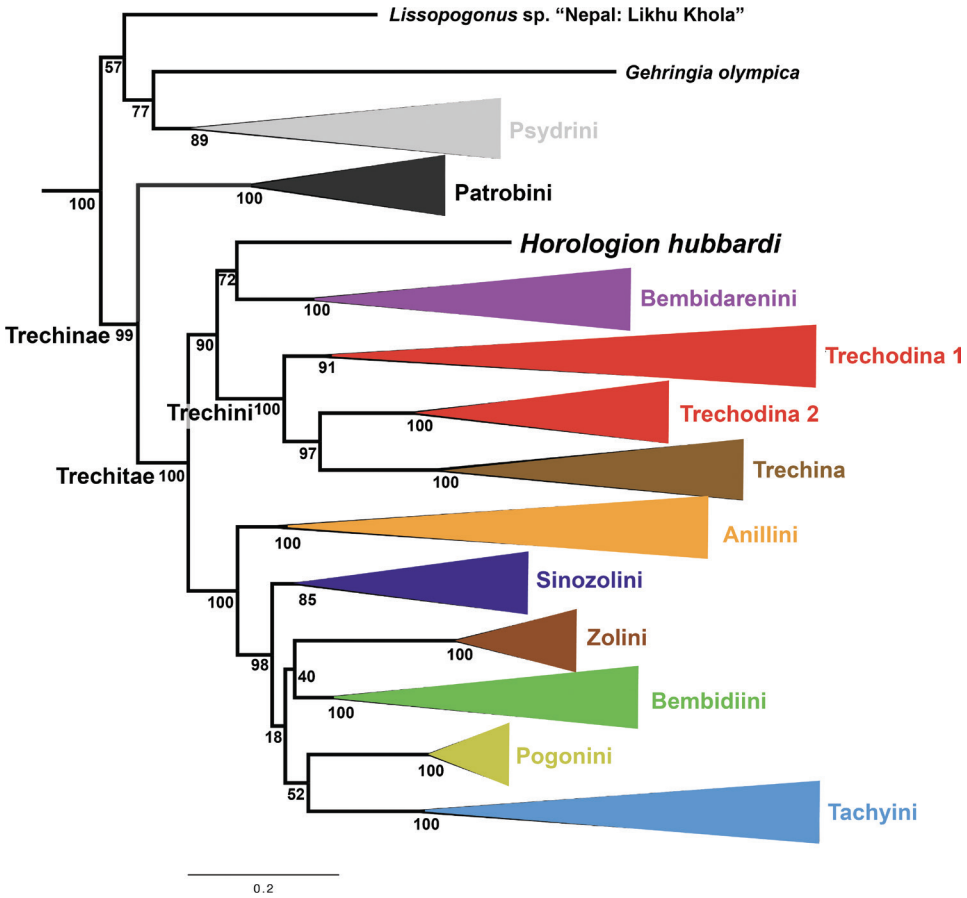

Figure 4. Summary of the maximum likelihood tree of Trechinae from IQ-TREE analysis of 8-gene concatenated matrix. Standard bootstrap support values are shown below nodes. For complete phylogenetic tree showing details within each clade, see Suppl. material 4.

except a subtriangular patch on vertex where the sculpticels are coarse and conspicuous. Occipital region (concealed by pronotum) smooth, demarcated from rough vertex by a curved marginal line. Vertex with anterior supraorbital seta present on each side (Fig. 12A); posteriorly with 3 to 5 pairs of moderately long inward-facing setae in a transverse row, none of which arise from a pore of comparable size to the anterior supraorbital setae. Frontal grooves weakly defined, shallow and short, ending at level of anterior supraorbital setae. Frontoclypeal suture weakly impressed, without carinae or horn like projection. Clypeus transverse, subrectangular, with four large fixed setae, outer pair erect and longer than inner pair; inner pair appressed, arising from smaller pores than outer pair (Suppl. material 3: fig. S3C); in addition to scattered background setae, two thin setae the same length as inner pair are present near anterior angles (Fig. 12A). Labrum transverse, similar in size and shape to clypeus; anterior margin slightly crenulate, protruding slightly forward at each setiferous pore inser-

	Horologion + Bembidarenini		*Horologion* + Bembidarenini + Trechini		Trechitae including *Horologion*	
	SBS	UFBoot, SH-aLRT	SBS	UFBoot, SH-aLRT	SBS	UFBoot, SH-aLRT
8 gene	72	100, 72.6	90	99, 96.6	100	100, 99.9
18S	6	79, 72.1	-11	88, 81.2	-11	100, 97.6
28S	15	66, 30.9	48	98, 96	80	99, 99.9
COI	-10	-91, -84.7	-17	-91, -84.7	-17	-96, -94.2
CAD2	-17	-76, -78.2	-17	-76, -78.2	-17	-88, -93.1
CAD4	-28	-43, 0	23	93, 92.5	25	95, 90.1
Wg	26	95, 89.2	-32	-95, -75.9	58	98, 92.6
Topo	20	-93, -96.8	-34	-88, -98.2	-34	-88, -98.2
MSP	-35	-38, -44.1	-35	-56, -80.7	24	73, 95.6
ArgK	-27	-99, -98.2	-59	-99, -98.2	-59	-98, -95.7

Figure 5. Support for and against our preferred hypothetical placement of *Horologion*. Black: clade present in maximum likelihood tree, SBS 90% or greater, UFBoot 95% or greater and SH-aLRT 80 or greater. Grey: clade present in maximum likelihood tree, SBS less than 90%, UFBoot less than 95% and/or SH-aLRT less than 80. Red: clade absent in maximum likelihood tree, most-supported contradictory clade with SBS 50% or greater, UFBoot 95% or greater and SH-aLRT 80 or greater. Pink: clade absent in maximum likelihood tree, most-supported contradictory clade with SBS less than 50%, UFBoot less than 95% and/or SH-aLRT less than 80.

	Horologion + Patrobini		*Horologion* + Psydrini		*Horologion* + Trechini		*Horologion* + Anillini	
	SBS	UFBoot, SH-aLRT	SBS	UFBoot, SH-aLRT	SBS	UFBoot, SH-aLRT	SBS	UFBoot, SH-aLRT
8 gene	-100	-100, -99.9	-100	-100, -99.9	-100	-100, -100	-100	-100, -100
18S	-11	-79, -72.1	-46	-100, -97.6	-22	-53, -80.9	-19	-92, -92.8
28S	-80	-99, -99.9	-80	-99, -99.9	-15	-97, -98.7	-48	-98, -96
COI	-13	-95, -81.4	—	—	-17	-96, -94.2	-27	-91, -84.7
CAD2	-25	-88, -93.1	-25	-88, -93.1	-25	-88, -98.3	-25	-76, -78.2
CAD4	-30	-95, -90.1	-25	-95, -90.1	-28	-99, -62.4	-56	-99, -94.6
Wg	-58	-98, -92.6	-58	-98, -92.6	-32	-100, -99.8	-32	-95, -89.2
Topo	-34	-93, -96.8	—	—	-34	-91, -96.1	-40	-93, -96.8
MSP	-29	-73, -95.6	-35	-73, -95.6	-29	-78, -85.4	-93	-100, -99.2
ArgK	17	86, 67.5	-17	-87, -88.3	-59	-99, -98.2	-100	-99, -98.2

Figure 6. Support for and against previously proposed placements of *Horologion*. Grey: clade present in maximum likelihood tree, SBS less than 90%, UFBoot less than 95% and/or SH-aLRT less than 80. Red: clade absent in maximum likelihood tree, most-supported contradictory clade with SBS 90% or greater, UFBoot 95% or greater and SH-aLRT 80 or greater. Pink: clade absent in maximum likelihood tree, most-supported contradictory clade with SBS less than 50%, UFBoot less than 95% and/or SH-aLRT less than 80. There were not sufficient sequences available for *COI* and *Topo* from our sampled Psydrini.

tion; six fixed apical setae present, decreasing in length from outer to inner pairs. Ventral surface of head with a long suborbital seta on each side, set just anterior to the arcuate gular impression; tentorial pits present at anterior end of gular sutures, with small slit like openings; ventral surfaces pubescent anterior to gular impression, except strip between gular sutures, which is also strongly microsculptured with coarse, small sculpticels; microsculpture also strong within gular impression, and along margins of maxillary grooves, weak elsewhere.

***Antennae*:** Length approximately half of body length (**AntL/ABL** = 0.51–0.53). All antennomeres pubescent, filiform; antennomeres I–X with a subapical ring of long

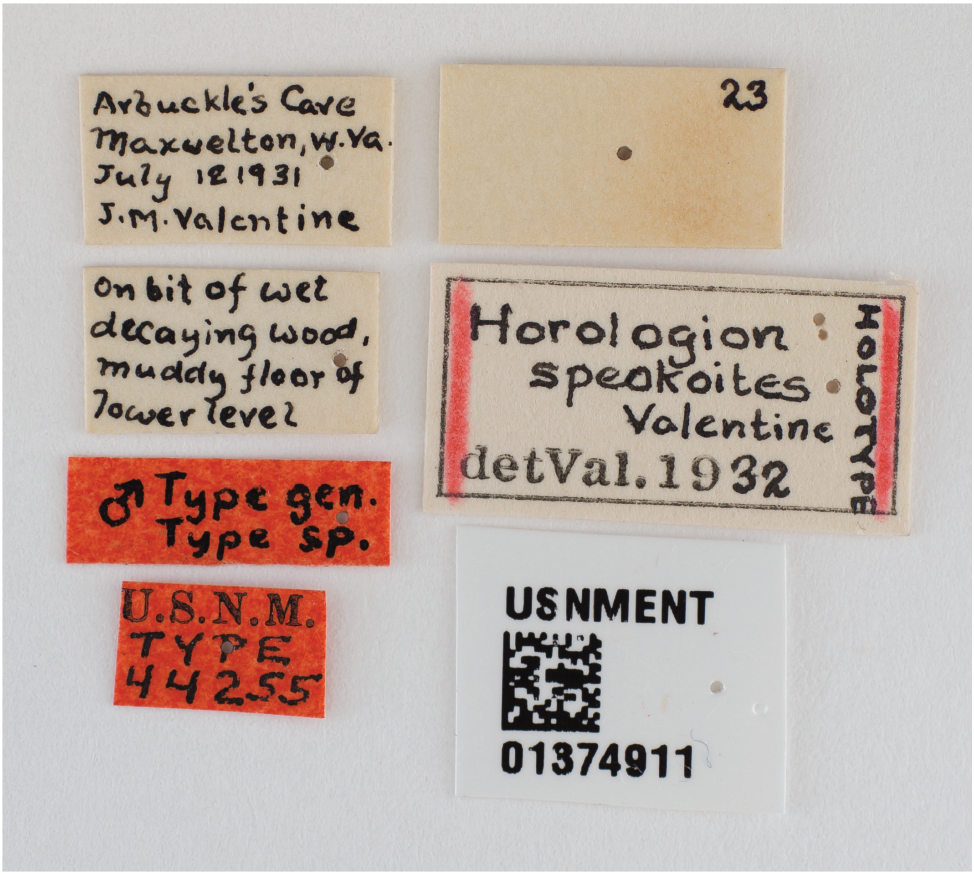

Figure 7. Labels of *Horologion speokoites* holotype, USNM.

setae, antennomere XI with a ring of long setae just beyond middle, and a crown of long setae at apex. Several small, circular pores scattered in apical half of antennomere XI, concentrated near apex. Antennomere I shorter and thicker than antennomeres II–X. Four apical antennomeres gradually increasing in width; antennomere XI largest, slightly longer than antennomere II and clearly longer than all other antennomeres; gradually tapered apically. Antennae similar in both sexes.

***Mouthparts*:** Mandibles with scrobal seta present; narrow and elongate, both similar in size and shape but differing in dentation: right mandible with prominent anterior retinacular tooth, terebral tooth, posterior retinacular tooth and molar tooth; left mandible without anterior retinacular tooth, with small terebral, posterior retinacular and molar teeth (Suppl. material 3: fig. S3A, B). Mentum and submentum separated by suture; submentum generally setose, with two pairs of long fixed setae, inner pair very long (the longest ventral setae of head); mentum transverse, surface glabrous except for two pairs of fixed setae, inner pair situated well behind mentum tooth; mentum shallowly biconcave, each concavity with a small irregular pit with numerous small pores; mentum tooth carinate, entire, long and acute (Fig. 12C). Labial

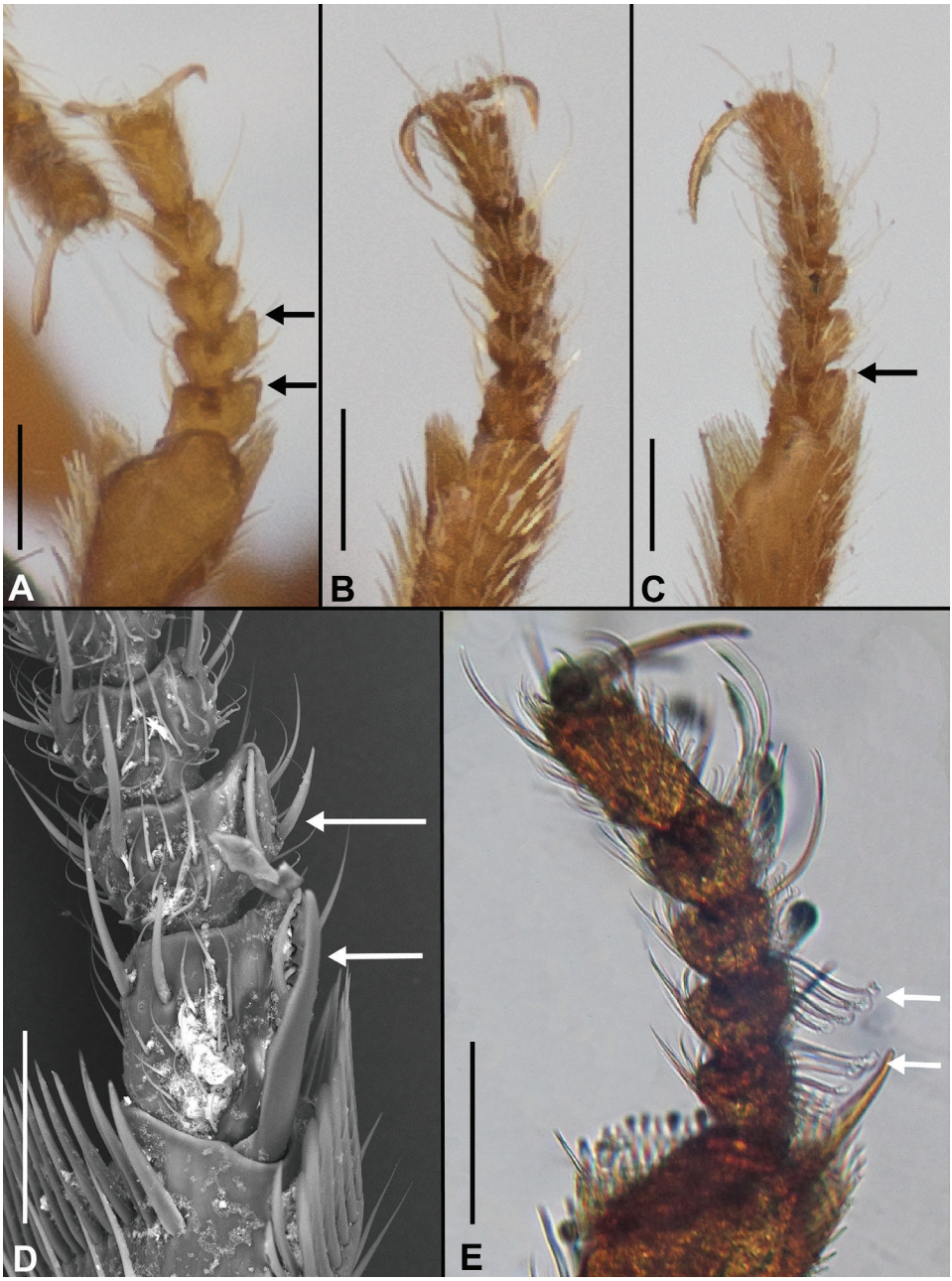

Figure 8. Protarsi of *Horologion* **A** *H. hubbardi* male holotype, dorsal aspect **B** *H. hubbardi* female paratype, dorsal aspect **C** *H. speokoites* male holotype, dorsal aspect **D** *H. hubbardi* male holotype, ventral aspect **E** *H. hubbardi* male holotype, dorsal aspect. Scale bars: 0.10 mm.

palps glabrous except for penultimate palpomere, which has four long setae; apical palpomere long, much narrower than penultimate. Ligula carinate medially, with distinct paraglossae; anterior margin between paraglossae with six setae, an outer pair of very

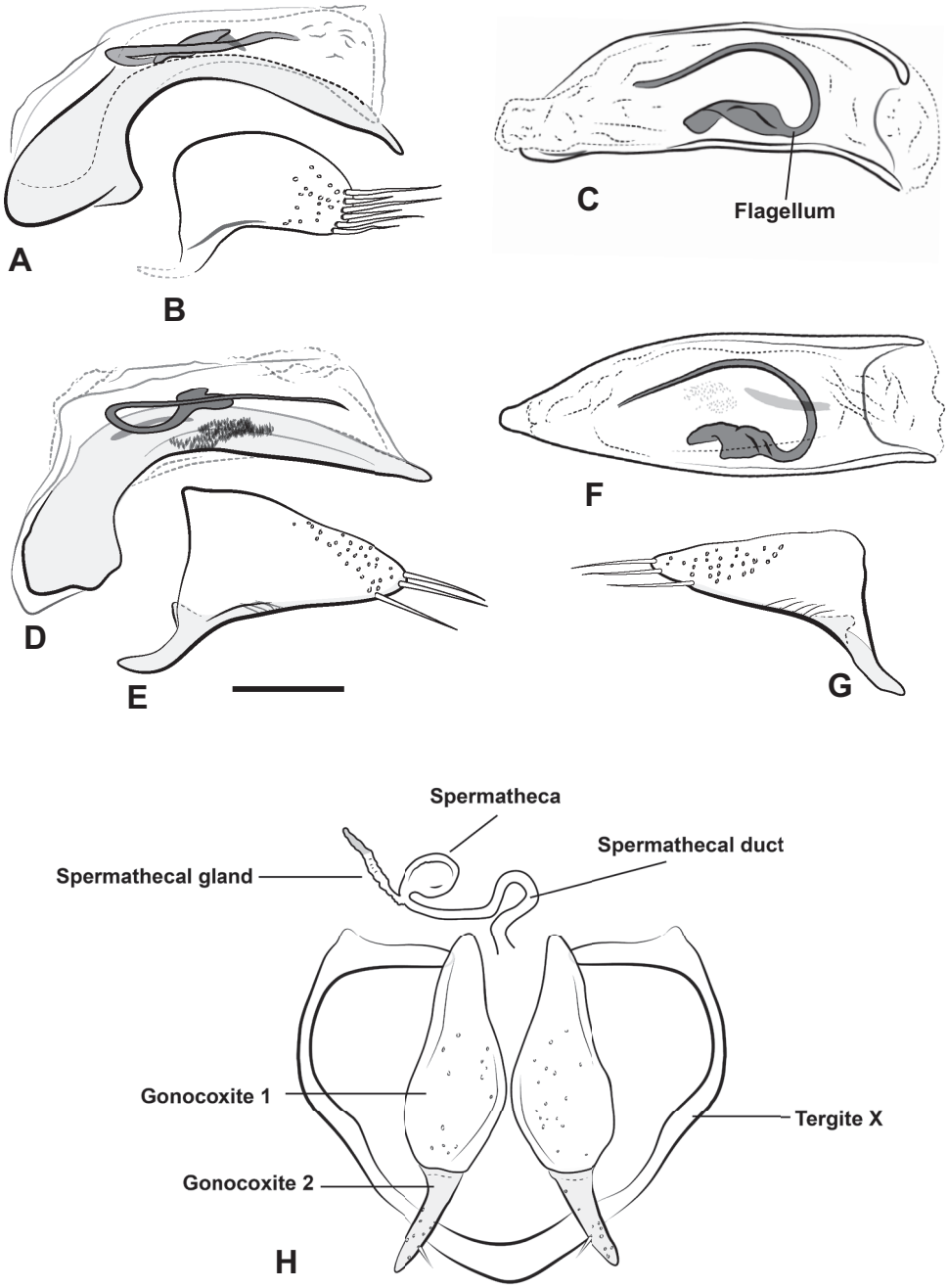

Figure 9. Genitalia of *Horologion* species **A** *H. speokoites* median lobe, left lateral aspect **B** *H. speokoites* left paramere, left lateral aspect **C** *H. speokoites* median lobe, dorsal aspect **D** *H. hubbardi* median lobe, left lateral aspect **E** *H. hubbardi* left paramere, left lateral aspect **F** *H. hubbardi* median lobe, dorsal aspect **G** *H. hubbardi* right paramere, right lateral aspect **H** *H. hubbardi* female genitalia, ventral aspect **A–C** after Valentine (1932). Scale bar: 0.1 mm.

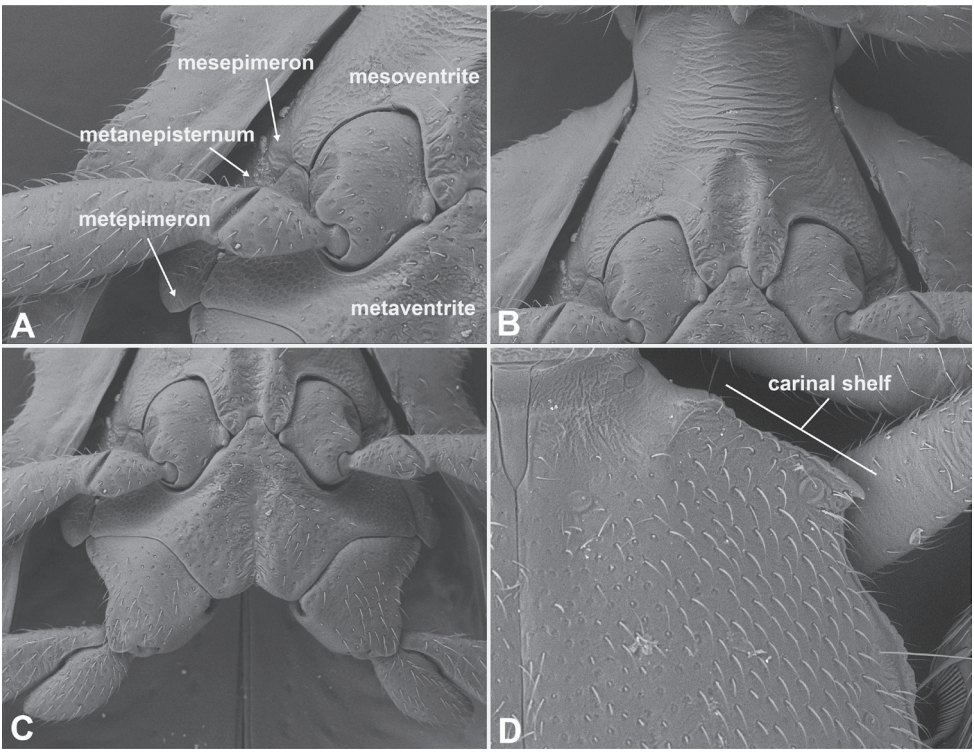

Figure 10. Scanning electron microscope images of *Horologion hubbardi* female paratype, voucher CWH-452 (VMNH) **A** detail of right side of pterothorax, ventral aspect **B** mesoventrite, ventral aspect **C** metaventrite, ventral aspect, abdomen removed **D** detail of humeral region of right elytron, dorsal aspect.

short setae, a submedial pair of moderately long fixed setae, and a medial pair of long fixed setae that are conjoined, arising from adjacent pores, appearing as one long seta except in SEM images; short outer pair not visible under a stereoscope at 100×, but visible in SEM images (Suppl. material 3: fig. S4A). Maxillary palps glabrous except second palpomere, which bears two setae on outer surface near apex; second and third palpomeres somewhat globular; apical palpomere narrow and elongate, but not truly subulate (basal width subequal to apical width of penultimate palpomere) (Fig. 12A).

***Prothorax*:** Pronotum small, narrower than elytra (**PW/EW** = 0.67) and less than one fourth body length (**PL/ABL** = 0.23); greatly narrowed posteriorly (**PbW/PW** = 0.39). Surface densely covered in light-colored setae, each set in a circular pore; setae whorled along midline: facing posteriorly in posterior half, medially in middle, and anteriorly in anterior half. Median longitudinal sulcus well impressed, but not reaching anterior or posterior margins. Lateral marginal bead lacking except for a short distance near lateral setae; otherwise, dorsal surface and hypomeron continuous. Posterior angles obsolete, without lateral setae; posterior impressions lacking. Posterior margin without bead, dorsal surface curved beneath itself, forming a smooth shelf that overhangs the mesothoracic pedicel. Prosternum (Fig. 12C) shorter than pronotum, ending

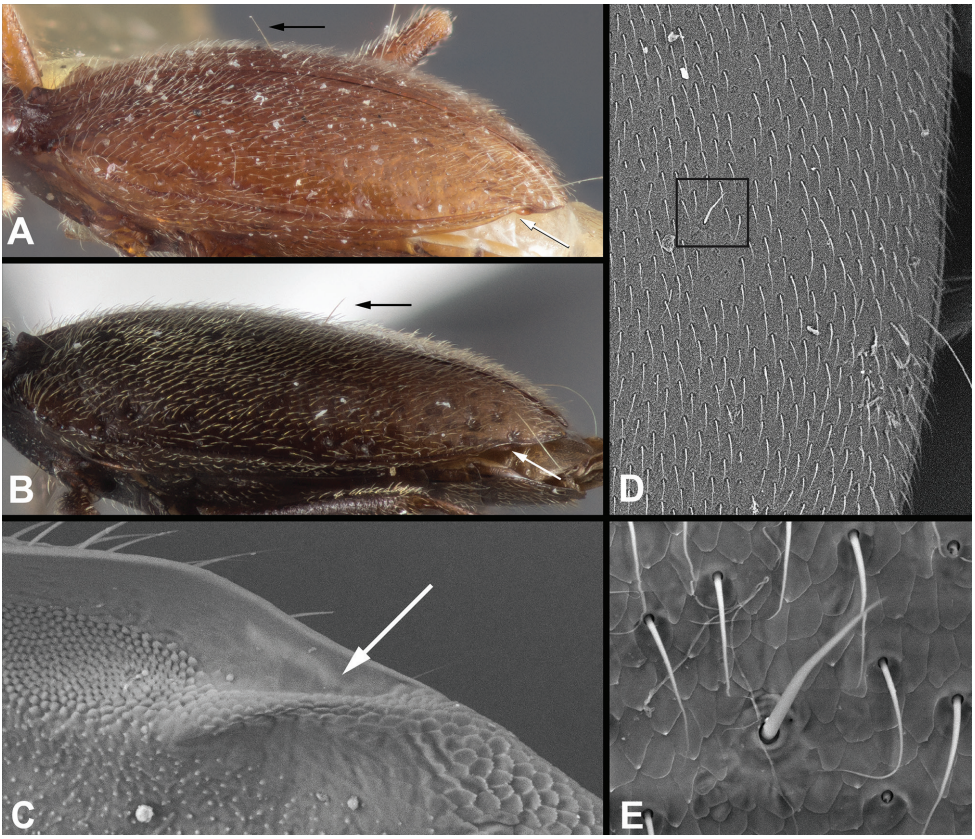

Figure 11. Elytral characters of *Horologion* species **A** *H. speokoites* holotype, elytra, oblique left lateral aspect **B** *H. hubbardi* paratype (CMNH), ditto **C** *H. hubbardi* paratype (VMNH), SEM detail of apex of left elytron, ventral aspect **D** *H. hubbardi* paratype (VMNH), SEM photo of right elytron, dorsal aspect **E** *H. hubbardi* paratype (VMNH), SEM detail of right elytron (area indicated by black box in **D**). Black arrows: discal setae, white arrows: plica.

anteriorly and posteriorly well before pronotal extent; setose medially; propleuron glabrous. Pleurosternal suture meeting hypomeron anteriorly behind anterior angles of prosternum, which are produced forward, overlapping hypomeron. Procoxal cavities bordered by raised margin anteriorly; closed posteriorly by propleuron narrowly joining intercoxal process (Fig. 12B). Intercoxal process elongate, acuminate posteriorly. Procoxae coarsely microsculptured, glabrous; protrochanters small, setose, with single large fixed seta near apex; femora and tibiae slender and setose, tibiae strigose on outer margin; outer margin declivitous in dorsal view, but without distinct notch (Fig. 8A). Inner margin of tibiae with large antenna cleaner of typical "grade B" (Hlavac 1971), i.e. with a sinuate longitudinal band of tightly packed setae within the channel; arrangement of terminal spurs anisochaetus, i.e. situated at opposite ends of the setal band; anterior spur stouter than posterior; one large clip seta present. Tarsi densely setose

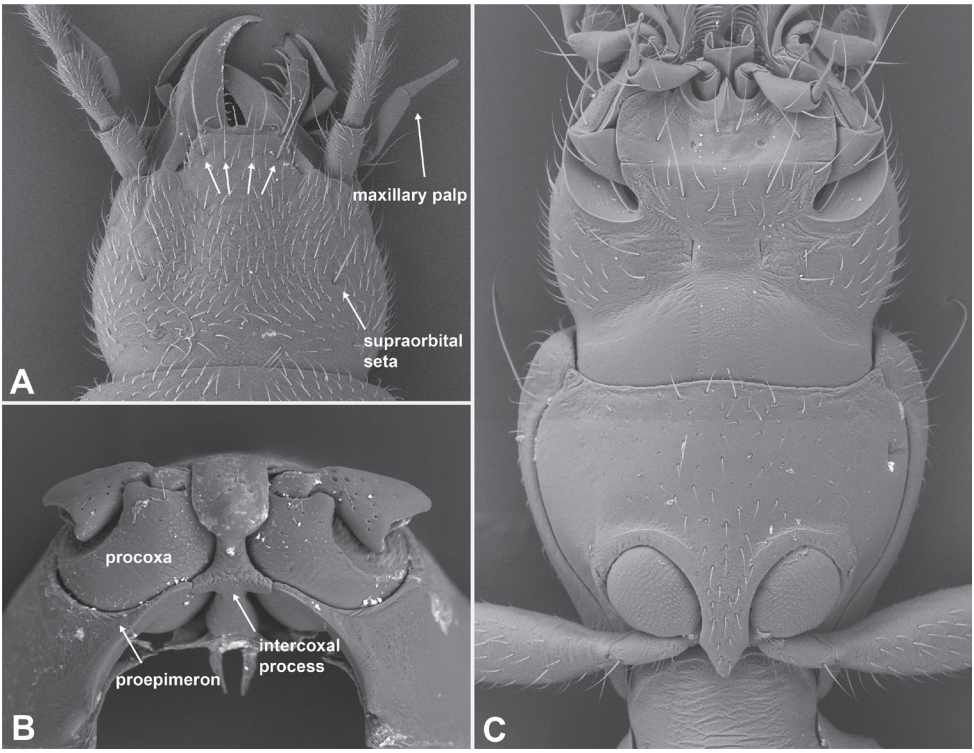

Figure 12. Scanning electron micrographs of the forebody of *Horologion hubbardi* **A** head of voucher CWH-452, dorsal aspect, clypeal setae indicated by white arrows **B** prosternum of non-type specimen, posterior aspect (head, pronotum and most of leg segments removed) **C** forebody of voucher CWH-452, ventral aspect.

and very short; protarsomeres 2–IV distinctly transverse, wider than long, each with a pair of long setae ventrally, ventral setae of protarsomere III longest and conspicuous; protarsomere IV with a thick medial ribbonlike seta that surpasses apex of tarsus; tarsal claws simple and evenly curved, without basal tooths or serrations, relatively elongate, longer than protarsomere V; males (Fig. 8A, C–D) with protarsomeres I and II asymmetrically expanded and dentate on inner margin, with a single row of at least seven adhesive setae on venter of inner dentate expansions; females with protarsomeres I and II symmetrical, not dentate and without ventral adhesive vestiture, protarsomere I slightly longer than wide, protarsomere II transverse, subequal to protarsomere III.

***Pterothorax*:** Elytra moderately long, length slightly more than half of **ABL**; scutellum very narrow and elongate (Fig. 10D). Elytra fused along suture for most of their length, narrowly separated in apical fourth. Dorsal surface evenly setose, with short light-colored setae each set in a deep circular pit; microsculpture weakly impressed, sculpticels irregularly shaped, scale like and longitudinally stretched. Humeri each bearing an angulate shelf, flanked proximally by a strong carina and ending in a prominent curved spine; lateral bead of elytra moderately crenulate beyond humeral spine for a short distance and smooth beyond that (Fig. 10D). Each elytron with a

parascutellar seta, four subhumeral lateral setae, two submedial lateral setae, three apical setae (two lateral and one discal, in the position of the "subapical seta" of Schmidt et al. (2021), umbilicate pore "8" of anilline taxonomists (Giachino and Vailati 2011; Sokolov 2013)), and one discal seta in third interval at about the level of fifth lateral seta (Fig. 11B, D); discal seta indistinguishable from background pubescence in low-magnification dorsal view but distinctly visible in oblique or lateral views (Fig. 11B) or at higher magnification (Fig. 11D, E); second subhumeral, second submedial, and posterior-most apical seta greatly elongate and filamentous. Ventral surface of elytra each with a well-developed lateral plica near apex, its surface strongly microsculptured with scale-like sculpticells (Fig. 11E). Mesoventrite (Fig. 10B) with coarse isodiametric microsculpture; narrow, much longer than metaventrite; extended anteriorly as a parallel-sided pedicel that extends beyond posterior extent of pronotum; surface of pedicel strongly rugose, with numerous transverse furrows; posterior half of mesoventrite with a medial longitudinal depression flanked by low, parallel carinae that coalesce anteriorly, each carina bearing a pair of long setae; medial depression extending posteriorly onto intercoxal process. Mesanepisternum and mesoventrite apparently fused, without discernible suture; mesocoxae conjunct, i.e., entirely enclosed by mesoventrite and metaventrite, mesepimeron not meeting mesocoxae (Fig. 10A). Mesocoxae with coarse scale-like microsculpture and sparse setae, each with a well-developed knob on inner margin; mesotrochanters densely setose but without apparent macrosetae; mesofemora and mesotibiae slender, setose except for glabrous area on posterior face of femora; inner face of mesotibiae strigose; apical half of mesotibiae with dense brush of coarse setae; apex of mesotibiae with adjacent pair of short spurs on posterior margin, barely extending past length of first mesotarsomere; mesotarsi of both sexes similar in form to female protarsi. Metaventrite (Fig. 10C) short, setose and coarsely microsculptured, with shallow medial depression; intercoxal process cleft posteriorly; metanepisternum and metaventrite separated by suture; metepimeron visible (Fig. 10A), overlapping first abdominal ventrite. Metacoxae setose, without apparent macrosetae; well separated, distance between them approximately equal to width of one mesocoxa; metatrochanters small, approximately equal in length to metacoxae, gradually narrowed apically, not strongly pointed; metafemora and metatibiae similar to those of mesothoracic legs, except metatibiae lack dense brush of setae; metatarsi more elongate than tarsi of other legs, metatarsomeres slightly longer than wide, gradually increasing in length from I to IV, V slightly longer than combined length of III and IV; medial ribbonlike setae on apex of metatarsomere IV narrower than on pro- and mesotarsi.

***Male genitalia*:** Relatively small (Length of ring sclerite / **ABL** = 0.16); ring sclerite similar to that of *H. speokoites* (Valentine 1932, fig. 13): yoke shaped, posterior margin produced as an obtuse angulation, narrowed anteriorly where sides join to form a short, flattened extension that is curved ventrally at its slightly asymmetrical apex. Median lobe of aedeagus (Fig. 9D) broad and lightly sclerotized, with entire dorsal margin and most of ventral face membranous; basal lobes and sides of ventral margin sclerotized and giving the organ a trough-like shape; ventral margin in lateral aspect slightly curved in proximal half, with membranous portion sagging below; apex small, extended a short

distance past membranous dorsal margin and appearing evenly rounded and symmetrical in dorsal aspect; median lobe not twisted from plane of basal lobes (Fig. 9F). Left paramere (Fig. 9E) relatively large and subtriangular, with numerous pores on dorsoapical margin and three apical setae. Right paramere (Fig. 9G) slightly smaller than left paramere, and more styliform, with numerous pores on dorsoapical margin and three apical setae. Internal sac of median lobe with well sclerotized flagellum surrounding a small spine and a ventral field of small sclerotized scales; flagellum rotated dorsally, so that in lateral aspect it appears as a complex folded structure (Fig. 9D); in dorsal aspect strongly curved (Fig. 9F), swollen and spiraled proximally at junction with sperm duct, abruptly narrowed beyond this region, very gradually tapering toward apex.

***Female genitalia*:** (Fig. 9H) Gonocoxite 2 narrow, moderately long and weakly curved, bearing numerous pores and a single short preapical seta on inner margin. Tergite X well-sclerotized and forming a subtriangular bridge behind gonocoxites. Spermatheca and spermathecal gland present; spermathecal duct relatively wide and moderately long, with an abrupt U-shaped bend proximally; spermatheca small and pouchlike; spermathecal gland long and narrow, arising from base of spermatheca near junction with duct.

Distribution. Known only from Williams Cave, in Bath County, Virginia. In the database of the Virginia Speleological Survey (VSS, https://www.virginiacaves.org/), this cave is number 2779.

Sympatry. Williams Cave is also home to the eyeless trechine *Pseudanophthalmus intersectus* Barr, which also occurs in two other nearby caves in Bath County (Virginia DCR-DNH data). An individual of this species was found in the same microhabitat as the female paratype of *H. hubbardi*, and members of the two species presumably occur in syntopy. The only other carabid beetle known from Williams Cave is the surface tachyine *Paratachys scitulus* (LeConte), a common and widespread species in eastern North America; one specimen was found in organic debris just inside the entrance in August 2022.

Natural history. Williams Cave is a large cave, with a surveyed length of 5.39 km (VSS data). The cave is shallow in relation to the overlying surface topology and is damp in places, with numerous ceiling drips and small pools. Most water in the cave is recharged through these ceiling drips. All specimens of *H. hubbardi* were found in or near small pools of water. Specimens were collected in March, August and September. Specimens from 2022 and 2023 were collected in somewhat distant sections of the cave, but *COI* sequences of the two are identical, suggesting they are not isolated. Immature life stages are unknown.

Species status justification. The differences in male protarsi (first and second protarsomeres asymmetrically expanded and dentate in *H. hubbardi*, only first protarsomere weakly dentate in *H. speokoites*), the form of the elytra (flattened, with prominent curved spines on the humeri in *H. hubbardi*, convex and with a small humeral carinal shelf without spines in *H. speokoites*), and the male genitalia, particularly the parameres (tapered and with 3 apical setae in *H. hubbardi*, broad and blunt with 6 apical setae in *H. speokoites*) are great enough to warrant recognition of the two as distinct taxa that are reproductively isolated. The two species are also geographically

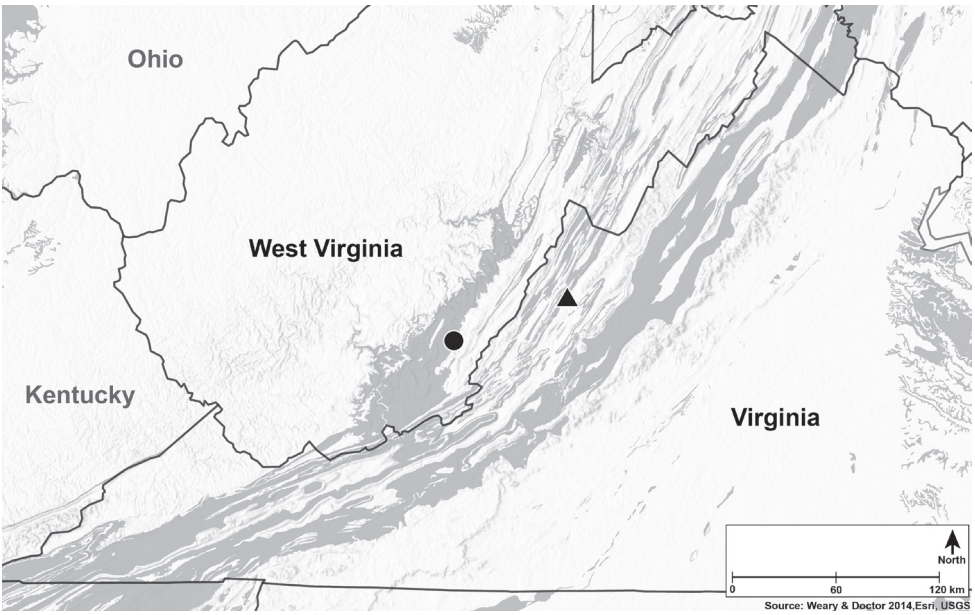

Figure 13. Distribution map of species of *Horologion*. Black triangle: *Horologion hubbardi*, black circle: *Horologion speokoites*. Grey shaded areas represent exposed karst (Weary and Doctor 2014).

isolated, occurring 70 air km distant and on the opposite side of several large ridges of noncarbonate rock with numerous peaks above 1200 m, indicating complete isolation of these blind subterranean beetles (Fig. 13).

Derivation of name. This species is named in honor of its discoverer, David A. Hubbard, Jr., in recognition of his important contributions to cave biodiversity and conservation. In addition to many significant collections of cave carabids in Virginia, notable discoveries by Hubbard include the single known specimen of the Chinese stygobiontic dytiscid genus *Sinodytes* (Spangler 1996) and a highly modified species of the pselaphine rove beetle genus *Mipseltyrus* that remains undescribed (C. Harden, personal observation).

Suggested vernacular name. "Hubbard's Hourglass Beetle".

Discussion

Molecular phylogenetics

Our molecular data strongly support the placement of *Horologion* within the supertribe Trechitae, and that it is most closely related to the tribes Bembidarenini and Trechini, but does not belong within either of them. Therefore, the placement of the genus within its own tribe, Horologionini, is warranted. A more specific placement of *Horologion* as sister to Bembidarenini is recovered in the 8-gene tree and half of the single gene trees, with moderate support in the 8-gene tree and weak support in the single gene trees. Previous

hypothesized placements of *Horologion* within Psydrini (Emden 1936; Ball 1960), Anillini (Erwin 1972) and Trechini (Valentine 1932) are not supported by our molecular data; evidence against those placements is strong (Fig. 6). The 8-gene analysis also provides strong evidence against a placement with Patrobini, as proposed by Jeannel (1949). There is some support for *Horologion* + Patrobini provided by *ArgK*; however, that clade is weakly supported (SBS = 16, UFBoot = 86, SH-aLRT = 67.5), and *ArgK* is known to be a problematic gene within carabids, due to the likely presence of paralogs (Maddison et al. 2019).

Morphology

The morphological evidence mirrors the placement of *Horologion* revealed by DNA sequence data: *Horologion* is clearly a trechite, likely belonging in a clade with the tribes Bembidarenini and Trechini, and is possibly the sister taxon to Bembidarenini. Synapomorphies for most large clades (including tribes) of Trechitae are unreported as there has been insufficient study of the distribution of morphological character states. However, we have surveyed representatives of all tribes through the review of literature and specimens available to us, and can corroborate most of the proposed synapomorphies for Trechitae and the clade containing Bembidarenini + Trechini (Maddison et al. 2019; Schmidt et al. 2021). We have also found evidence for additional synapomorphies of various clades. The results of this review are presented below from higher to lower taxonomic placement, and are summarized in Fig. 14.

Evidence that *Horologion* is a trechite

Horologion possesses character states of the sterna (conjunct mesocoxae, closed procoxae, visible metepimeron overlapping the first abdominal ventrite) and appendages (protibiae with "Grade B" antenna cleaner (Hlavac 1971) and anisochaetus terminal spurs) that indicate it is a "middle grade" carabid ("Carabidae conjunctae"), and the presence of a seta in the mandibular scrobe excludes it from Harpalinae (Schmidt et al. 2021). The sternal characters, along with dorsally visible antennal insertions and the absence of any fossorial leg adaptations, exclude it from Scaritinae, with which it shares a pedunculate junction between the prothorax and mesothorax. Placement within the subfamily Trechinae is supported by the presence of a dorsally open median lobe, an autapomorphy proposed by Schmidt et al. (2021). Further, *Horologion* possesses three of the four proposed autapomorphies of the supertribe Trechitae (Maddison et al. 2019; Schmidt et al. 2021): setose antennomere 2, dentate male protarsomeres, and (in our interpretation) a 4+2+2 arrangement of umbilicate punctures. The only trechite synapomorphy lacking in *Horologion* is the subequal scape and pedicel; the scape is distinctly shorter than the pedicel in *Horologion*. This is likely due to elongation of the antennae associated with adaptation to a subterranean environment, a common morphological change seen in cave insects (Moldovan 2012; Faille et al. 2013).

One of the key discoveries we have made is that the basal male protarsomeres of *Horologion* are dentate, as in most trechites. The previous suggestions that *Horologion*

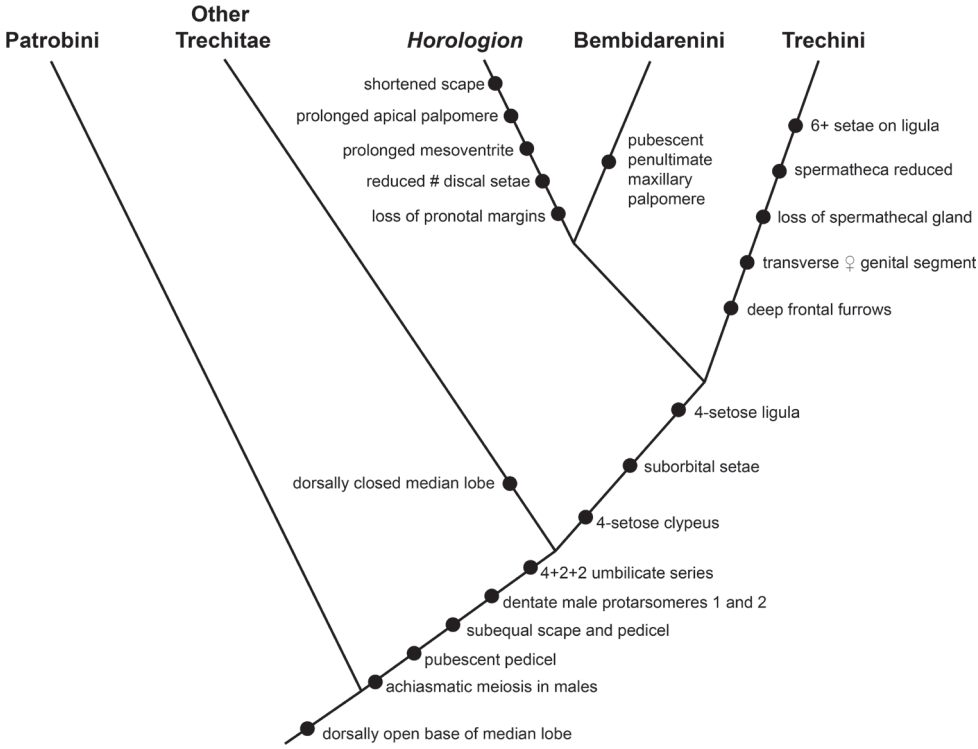

Figure 14. Phylogenetic tree of a portion of Trechinae showing proposed synapomorphies of clades. Topology is that of Fig. 4.

was not a trechite (van Emden 1936; Jeannel 1949) were based solely on Valentine's description of *H. speokoites*, in which the male protarsomeres were erroneously described as not dentate. The first protarsomere of *H. speokoites* is much less strongly dentate than in most trechites, including the new species *H. hubbardi*, but it is dentate (Fig. 8C). The denticle is not apparent when viewed at certain angles, including the one Valentine illustrated, which is the view provided by the orientation of the permanently mounted left foreleg of the holotype. The second protarsomere of *H. speokoites* is not dentate. The degree of enlargement and number of dilated protarsomeres varies within trechites, and species with males whose protarsomeres are identical to those of females are known in distantly related tribes such as Anillini (Sokolov and Kavanaugh 2014), Tachyini (Boyd and Erwin 2016) and Trechini (Tian et al. 2023). Thus, the morphological traits of *Horologion* are fully consistent with it being a member of Trechitae (Fig. 14).

Evidence that *Horologion* + Bembidarenini + Trechini form a clade

The two character states considered by Schmidt et al. (2021) to be synapomorphies of Bembidarenini + Trechini are also found in *Horologion*: a quadrisetose clypeus and a suborbital seta on each side of the head. Due to the densely pubescent dorsal surface of

the head, the four fixed clypeal setae in *Horologion* are not at first obvious. The outer pair is conspicuous, being large and erect. The inner pair is more difficult to see in a dorsal aspect; the inner setae are shorter and slightly smaller in diameter than the outer pair, and are more decumbent. However, the same is true of all species of Bembidarenini examined (three species of *Bembidarenas*, four species of *Argentinatachoides*, three species of *Andinodontis*, and 24 species of *Tasmanitachoides*), in members of Trechini (e.g. Ortuño and Barranco 2013 fig. 4c, and 2015 fig. 3f), and in the extinct genus *Balticeler* from Baltic amber, which is likely a member of the stem group of the *Horologion* + Bembidarenini + Trechini clade (Schmidt et al. 2021, fig. 6). We have confirmed the presence of four fixed clypeal setae in five specimens of *Horologion*: the holotype of *H. speokoites* (which Valentine also illustrated as having four clypeal setae (Valentine 1932 fig. 8)), the holotype and two female paratypes of *H. hubbardi*, and one individual of *H. hubbardi* that was found dead and studied with SEM photography (Suppl. material 3: fig. S3). The other individuals of *H. hubbardi* that were found dead were missing all of the clypeal setae, and only the outer pair of pores could be seen with a stereoscope.

The four fixed clypeal setae are not always arranged in the same way in members of the *Horologion* + Bembidarenini + Trechini clade. In most, including *Horologion*, the pores are set more or less in a straight line across the clypeus, well removed from its anterior margin. In *Tasmanitachoides*, the inner pair is situated close to the anterior margin, whereas the outer pair is in the typical position to the posterior of these. The examined individuals of *Tasmanitachoides hobarti*, *T. murrimbidgensis*, *T. erwini*, and *T. kingi* also have 1 or 2 additional clypeal setae on each side present near the anterior lateral angles of the clypeus. *Horologion* also possesses a third pair of clypeal setae in the same position, which are the same length as the inner pair, though smaller in diameter. The presence of only one pair of fixed clypeal setae has been reported in a small number of species of Trechini (Belousov 1998; Faille et al. 2023; Naitô 2023), and in the recently described genus *Baehria*, three to five fixed setae are present on each side (Faille et al. 2023).

Schmidt et al. (2021) state that a quadrisetose clypeus is known in Trechinae only in the tribes Bembidarenini and Trechini. However, several species in the tribe Anillini also have a quadrisetose clypeus. The Mexican cave-dwelling genus *Mexanillus* is illustrated as having four fixed setae on the clypeus (Vigna Taglianti 1973), and four clypeal setae are distinctly shown in the illustrations of the African genus *Bafutyphlus* (Bruneau de Miré 1986), most species in the Greek genera *Prioniomus* and *Parvocaecus* (Giachino and Vailati 2011), and the New Zealand genera *Hygranillus* Moore (Larochelle and Larivière 2007, fig. 51), *Nesamblyops* (Larochelle and Larivière 2007, figs. 52, 136), *Pelodiaetodes* (Sokolov 2015), and *Pelodiaetus* (Sokolov 2019). We have confirmed the presence of four fixed clypeal setae in specimens of *Nesamblyops*, and the holotype of *Hygranillus kuscheli* Moore was confirmed by R. Leschen to have four fixed clypeal setae. Considering the distant relationship of Anillini to Bembidarenini and Trechini indicated by molecular results, and the typical state of a bisetose clypeus in the majority of Anillini, the quadrisetose clypeus of these anilline taxa most likely represents one or more independent origins. Alternatively, some of these taxa might not be anillines. *Nesamblyops* is the only one of these that has been sampled for molecular phylogenetic

studies, and has been recovered as sister to the remaining Anillini sampled (Andújar et al. 2016; Maddison et al. 2019). This homoplasy of the quadrisetose clypeus detracts somewhat from its strength as a synapomorphy of *Horologion* + Bembidarenini + Trechini, but in light of the ubiquity of the character state and the consistent arrangement of the pores (with the exception of *Tasmanitachoides*), we suggest that these setae are homologous within the *Horologion* + Bembidarenini + Trechini clade.

An additional character state that supports the *Horologion* + Bembidarenini + Trechini clade is the presence of four or more fixed setae on the ligula. *Horologion* and the four genera of Bembidarenini have four large apical setae on the ligula (plus two small inconspicuous setae) (Suppl. material 3: fig. S4) and members of Trechini possess six or more. The presence of four or more ligular setae is likely derived, as nearly all Geadephaga possess a bisetose ligula. The only other trechites that are known to have more than two setae on the ligula are the sinozolines *Chaltenia* (Roig-Juñent and Cicchino 2001) and *Phrypeus* (our observations), and some members of Tachyini (Jeannel 1941; Erwin 1973), which probably represent independent origins of the character state.

Evidence that *Horologion* + Bembidarenini form a clade

In our molecular results, support for a *Horologion* + Bembidarenini clade is moderately strong, as the clade is present in half of the single gene trees as well as the 8-gene tree (with SBS of 72)%. Morphological support for the clade is lacking, as most of the characters shared between *Horologion* and Bembidarenini (quadrisetose clypeus, quadrisetose ligula, suborbital setae, dorsally open median lobe of the aedeagus) are likely plesiomorphic in the *Horologion* + Bembidarenini + Trechini clade. Two characters included in the description of Bembidarenini (Maddison et al. 2019) are apparently derived within the *Horologion*+Bembidarenini+Trechini clade: a pubescent penultimate maxillary palpomere and unique form of the frontal furrows (shallow, closer to the eyes than to each other). *Horologion* lacks the first of these, and the latter is difficult to judge since the eyes are absent.

Evidence that *Horologion* does not belong in Trechini

The female genitalia of *Horologion* and bembidarenines are similar in structure, but we view the evidence provided by these similarities to be weak, as there is extensive variability present in the relatively few trechites studied. However, these characters provide some evidence that the two taxa are not members of Trechini. *Horologion* and bembidarenines possess subtriangular genital segments with elongate second gonocoxites that bear few setae, and all have a well-developed spermatheca that bears a narrow spermathecal gland. Spermathecae in trechites have not been thoroughly studied, but the form possessed by *Horologion* and the bembidarenine genera (compact, and with an attached gland) has been proposed as a derived form (Belousov and Kabak 2005). If so, it has apparently also evolved independently in the tribes Anillini (Sokolov 2013), Bembidiini (Maddison 1993; Neri et al. 2011), and Tachyini (Erwin 1973; Liebherr 2021).

In contrast, members of Trechini possess a strongly transverse female genital segment with short gonocoxites, the spermathecal gland has been lost, and the spermatheca is reduced in all Trechina taxa studied to date, being baggy and poorly defined or entirely absent (Schuler 1971; Deuve 1993; Toribio and Rodríguez 1997; Liebherr and Will 1998; Townsend 2010; Ortuño and Novoa 2011; Reboleira and Ortuño 2014; Yahiro 2014; Ortuño and Barranco 2015; Vrbica et al. 2018). We have also observed the lack of a spermatheca in three species of Appalachian *Trechus* in both *Trechus* (*s. str.*) and *Trechus* (*Microtrechus*). We have not found descriptions of the spermatheca in any genera of the subtribe Trechodina except *Perileptus*, in which the spermatheca is present and distinct but lacks a gland (Ortuño 1991; Yahiro 2014). Determining the character states in other members of this subtribe would help test the hypothesis that loss and/or reduction of these structures is a synapomorphy of the tribe; we have explored the spermatheca of representatives of all tribes of Trechitae, either by examining published descriptions or through dissection of our own specimens, and reduction of the spermatheca in Trechini appears to be a derived state within the supertribe.

Relationships of Lovriciina

The subtribe Lovriciina, placed in Bembidiini *sensu lato* by Giachino et al. (2011), was hypothesized as a possible relative of *Horologion* by Maddison et al. (2019). The group consists of four rare species in three genera (*Lovricia* Pretner, *Neolovricia* Lakota, Jalžić and Moravec, and *Paralovricia* Giachino, Guéorguiev and Vailati) distributed in Croatia and Bulgaria. All lovriciines are small (less than 3 mm), eyeless, elongate, slender, and known exclusively from caves. As with *Horologion*, the systematic placement of lovriciines has been enigmatic. Giachino et al. (2011) proposed six synapomorphies of lovriciines: (1) extremely long and narrow apical palpomeres, (2) absence of fixed setae at hind angles of pronotum, (3) absence of discal setiferous punctures on elytra, (4) umbilicate series consisting of nine punctures, (5) absence of apical recurrent stria on elytra and (6) uniquely expanded and spinose apex of mesotibiae. Based on the character states in Valentine's (1932) description, *H. speokoites* appeared to share the first five of these, and Maddison et al. (2019) hypothesized that Lovriciina may be the sister group to *Horologion*. In light of our study, the evidence for this relationship is rather weak. The form of the maxillary palpomeres is strongly homoplastic and not a reliable indicator of relationship (Maddison et al. 2019), and the apical palpomere is much thinner in lovriciines than in *Horologion*. Loss of the posterior lateral pronotal setae has occurred in numerous trechites, and in *Horologion* it is accompanied by the complete obliteration of the marginal bead and hind angles, both of which are present in lovriciines. The shared lack of elytral discal setae no longer applies in light of our finding that a discal seta is present on each elytron in both species of *Horologion*. The umbilicate series is arranged in the same manner in *Horologion* and lovriciines, but an identical arrangement is found in many anillines (Giachino and Vailati 2011) and in trechines that lack an apical recurrent stria (Tian et al. 2023); the homology of this character state is doubtful.

There is some evidence that lovriciines possess a quadrisetose clypeus and suborbital setae, the two synapomorphies of *Horologion* + Bembidarenini + Trechini (Fig. 14): the clypeus is described (but not illustrated) as having four fixed setae in *Paralovricia beroni* (Giachino et al. 2011) and *Neolovricia ozimeci* Lakota, Jalžić and J. Moravec (Lakota et al. 2009), and in the photograph of a live individual of *Lovricia aenigmatica* Lakota, Mlejnek and Jalžić in Hlaváč et al. (2017, fig. 67a), a long suborbital seta appears to be visible. The presence of four clypeal setae in the slide-mounted holotype of *P. beroni* was confirmed by Rostislav Bekchiev (National Museum of Natural History, Bulgarian Academy of Sciences), but they reported that suborbital setae could not be found. Two of the possible synapomorphies of Trechini are also found in the female paratype of *P. beroni*: a baggy, reduced spermatheca and lack of a spermathecal gland. However, some anillines with a reduced spermatheca lack an attached gland (Giachino and Vailati 2011 fig. 109; Magrini 2013), so this is yet another character within trechites subject to homoplasy. Lovriciina might belong near the *Horologion* + Bembidarenini + Trechini clade, but current knowledge of lovriciine morphology is too sparse to judge the character states that would support such a relationship.

How aberrant is *Horologion*?

From the beginning, *Horologion* was tagged as a strange and confusing carabid. Valentine (1932) listed six "aberrant characters" of *Horologion*: (1) shape of pronotum, with reduction of pronotal margins and loss of posterior marginal setae; (2) reduced number of elytral striae including loss of apical recurved stria; (3) denticulate margins and humeral carina of elytra; (4) lack of discal setae on the elytra and reduced number of umbilicate pores; (5) apical comb of the front tibiae; (6) shape of protarsomeres. In light of current knowledge, none of these characters are especially unusual within Carabidae. (1) and (2) are known in members of several other trechite genera (e.g. *Tianotrechus* (Tian et al. 2016)), although the loss of pronotal margins is uncommon. The 'denticulate margins' of (3) are seen in most anillines, some tachyines and many trechines; the humeral carinae of the elytra are seen in some bembidarenines and some members of the trechine genus *Stygiotrechus*. As we have pointed out already, the lack of discal setae (4) is not accurate. Valentine's interpretation of the umbilicate series as consisting of only six setae (4) prevented him from recognizing it as typical of many trechites. The apical comb of the front tibiae (5) is also typical of trechites, though is perhaps more prominent than in most. The shape of protarsomeres (6) in *H. speokoites* was not accurately described by Valentine, who did not notice that the first protarsomere is dentate on the inner margin. Having only a single male, Valentine was unaware that the protarsomeres of female *Horologion* are transverse as well (Fig. 8B), and therefore misinterpreted the third and fourth protarsomeres as expanded. The surprising hypothesis that *Horologion* belonged in Psydrini, proposed by van Emden (1936) and followed by Ball (1960), along with the unfortunate error on the state of the mesocoxae in the latter, surely played a role in clouding the proper placement of the genus.

The most notable characters of *Horologion* are those of the thoracic ventrites and the humeral carinae. The procoxae are placed well anterior to the posterior margin of the pronotum, and the mesoventrite is elongate, extending well anterior of posterior margin of pronotum. The metacoxae are widely separated (Fig. 10C), an unusual character in Carabidae. Modified humeri, with spines or carinae, are rare in the subfamily Trechinae. In the Bembidarenini, some members of the genus *Andinodontis* have short carinae on the humeri and many members of *Tasmanitachoides* have the humeral margin produced into a long carina extending onto the elytral disc. Some species of Tachyini have a humeral projection and a prolonged carina on the elytral disc similar to that of *Tasmanitachoides* (Terada et al. 2013). In Trechini, the Palearctic genera *Italaphaenops* and *Casaleaphaenops* possess a single smooth spine on each humerus (Ghidini 1964; Tian et al. 2021), and species of *Stygiotrechus* in the *morimotoi* and *unidentatus* groups have humeri that are similar to the form seen in *H. hubbardi*, with a raised carinate shelf terminating in a recurved tooth (Uéno 1969, 1973, 2001). Presumably these varied humeral processes represent independent origins, especially considering the different humeri of *H. speokoites*. No function that might explain such a convergence has been observed.

Natural history

Horologion has long been recognized for its extreme rarity. In the most recent faunal treatment of West Virginia cave invertebrates (Fong et al. 2007), *H. speokoites* is even considered "likely extinct." Although Valentine and subsequent authors (e.g., Barr 1969; Fong et al. 2007) indicated that great effort was made to obtain more specimens, there are few published records of these attempts. The only subsequent published records of beetle collecting at Arbuckle Cave are those of West Virginia University biology professor A.M. Reese in 1932 (summarized in Price and Heck 1939 p.114) and the French cave biologist Henri Henrot, who visited the cave in 1946 as part of an extensive collecting tour of Appalachian caves (Henrot 1949). Other published records of collections in Arbuckle from the 1960s and 1970s exist for isopods (Schultz 1970) and amphipods (Holsinger 1978), respectively. We found unpublished evidence of additional beetle collecting trips in the Carnegie Museum of Natural History. Specimens of *Pseudanophthalmus grandis* Valentine were collected in Arbuckle Cave on 22 September 1950 (W.B. Jones and J.M. Valentine), 13 June 1963 (T.C. Barr), and 18 July 2009 (R. Davidson, R. Acciavatti, R. Ward, and E. Saugstad). In the collecting notes of T.C. Barr, we found documentation of two additional visits he made to Arbuckle Cave on 11 April 1957 and 10 August 1958.

The rarity of *Horologion* has most frequently been explained by its likely preference for a microhabitat that is impossible for humans to visit, such as deep soils or epikarst (Barr 1969; Culver et al. 2012). The legs of *Horologion* do not possess any fossorial adaptations, and we consider it doubtful that *Horologion* actively excavates passages through deep soils. A constricted pro-mesothoracic junction such as that seen in *Horologion* has been interpreted as an adaptation for burrowing behavior (Evans 1991; Sokolov 2013), but it could also be an adaptation for maneuvering through the tight honeycombed rock layers of the epikarst. The relatively short length of the fixed setae and appendages

in *Horologion* also suggest that the beetles live in smaller interstices rather than the large open caverns where they have been collected (Moldovan 2012; Faille et al. 2013).

The finding of all specimens of *H. hubbardi* in or near drip pools supports the hypothesis that the terrestrial epikarst is the primary habitat of *Horologion* (Culver et al. 2012; Culver and Fong 2018), and the fact that all but two of the specimens were found dead supports the hypothesis that caves are not a hospitable habitat for *Horologion*. Drip pools in caves, formed by water percolating out of the epikarst, have long been known to harbor rare stygobionts; these water bodies are disconnected from other cave water features such as streams and phreatic groundwater aquifers, and the aquatic fauna of the two habitat types can be quite different (Holsinger 1978). That stygobionts enter drip pools by falling from the ceiling has been demonstrated through direct sampling of ceiling drips using special funnel collectors; in addition to capturing aquatic animals, these drip collectors have captured terrestrial invertebrates, including carabid beetles (Pipan et al. 2008).

The possible variety of terrestrial epikarstic microhabitats is visualized in fig. 10 of Eagle et al. (2015), who hypothesize that epikarstic voids "function as a series of cascading and leaking reservoirs that fill from the top and drain from the bottom." Terrestrial microhabitats along the margins of such fluctuating bedrock reservoirs would be bare and seasonally disturbed, not unlike the sand and gravel stream margins on which other relict trechites occur, including all species belonging to the tribes Sinozolini and Bembidarenini (Maddison et al. 2019). The voids in epikarst are also subject to flooding, and *Horologion* possesses morphological features that could be adaptations for surviving inundation. The large elytral plica (Fig. 11A–C) and interlocking promesothoracic junction would serve to seal the spiracles, and the dense pubescence and strong microsculpture could repel water or retain a plastron of air bubbles (Ortuño and Jiménez-Valverde 2011). The widely separated metacoxae could also represent an adaptation for bracing in place on substrate or in crevices during periods of flooding.

Distribution and biogeography

The two caves from which *Horologion* have been collected have little in common. Arbuckle Cave (written as "Arbuckle's Cave" by Valentine [Fig. 7]) is small, with a single passage that is approximately 78 m long (West Virginia Speleological Survey [WVASS] data), yet it is among the most biodiverse caves in the Greenbrier Valley (Culver and Fong 2018). In contrast, Williams Cave is much larger, with a surveyed length of over 5 km (VSS data), and it is home to a relatively depauperate fauna (Virginia DCR-DNH data). Both caves are relatively shallow in relation to surface topology, with open cow pastures on the surface (Fig. 3A, E), and the water in both is primarily recharged through epikarst. They are both located in river valleys, although the valley of the Greenbrier River (Arbuckle Cave) is much larger than that of the Cowpasture River (Williams Cave). The Greenbrier Valley has the character of a high plateau, while the Cowpasture is tucked between steep mountain ridges. The extent of karst in the two valleys is quite different as well, with the Greenbrier containing larger continuous

deposits than in the Valley and Ridge province of western Virginia, where karst is largely limited to narrow strips between the ridges of resistant rock (Fig. 13).

Valentine's (1932) characterization of Arbuckle Cave and other Greenbrier Valley karst features as "caverns in early stages of formation" is incorrect. Carbon-14 dating of vertebrate fossils collected in Greenbrier Valley caves has estimated dates ranging from 35,960 to 11,350 years before the present (Garton and Grady 2018); the caves themselves are likely at least two orders of magnitude older than that (White 2018a). Arbuckle Cave in particular is likely one of the oldest caverns in its vicinity, given its development in upper strata that have largely eroded away in this part of Greenbrier County. Stratigraphically, Arbuckle Cave is in Greenbrier Group Patton Limestone of Mississippian age. The cave sits above, but is not connected to, the enormous Great Savannah Cave System, one of the longest caves in the United States, with a surveyed length of over 85 km (WVASS data). Most of the surface drainage around Arbuckle Cave is now captured by this system at the contact between the MacCrady Shale and Hillsdale Limestone. Arbuckle Cave has no surface streams that feed into it, and all the water in the cave is recharged through the epikarst, creating flowstones (Fig. 3F) and drip pools.

Williams Cave is located in Bath County, Virginia, in the valley of the Cowpasture River, a small, meandering tributary of the James River. Numerous caves exist in the valley, including several that, like Williams, exceed 5 km in surveyed length; the longest of these is approximately 10.3 km long (VSS data). Williams is largely developed in the Devonian aged Little Cove Member of the Licking Creek Limestone of the Helderberg Group, with lower portions of the cave being developed in the Cherry Run Member of the Licking Creek Limestone (Haynes et al. 2014). The cave is wet in places, with numerous active ceiling drips and pools (Fig. 3C), but does not currently have active stream passages; as in Arbuckle, most of the water in Williams is likely recharged through the epikarst. Diverse microhabitats exist in the cave, and include bare rock crawlways with extensive calcite formations, dusty avenues historically mined for saltpeter, high-ceiling rooms floored with large blocks of mud-covered breakdown, slopes of bare talus, and passages covered in wet, sticky mud 50 cm deep or more. In lower sections where cave passages penetrate the Cherry Run Member, pockets of wet gravel containing numerous bivalve fossils are found. The female molecular voucher of *H. hubbardi* was collected in one such site (Fig. 3B), whereas all other specimens were found in muddy passages (Fig. 3C).

Williams Cave has had a much more complex history of human use than Arbuckle. Evidence of Native American visitation exists in the cave, including pine torch fragments that have been carbon dated to between 995 and 1060 CE, and the cave was also mined for saltpeter during the American Civil War (Faulkner 1988). During the second World War, Williams Cave was one of several in the Appalachian region that was blasted shut by the U.S. Army as part of military practices (Douglas 1964). The entrance was re-dug by members of the University of Virginia caving club in 1974, including David Hubbard, Jr., who collected the first specimen of *H. hubbardi* in 1991, during an early bioinventory of the cave.

The location of the two *Horologion* caves on opposite sides of the high mountains in the Valley and Ridge province on the Virginia-West Virginia border is noteworthy;

Barr (1985) considered this region an important refugium for ancestral Trechini, based on the distributional patterns of *Pseudanophthalmus* species and the region's distinction as a high elevation area that would have been suitably cold and wet over a long period without being subject to glaciation. The common ancestor of the two *Horologion* species likely also inhabited this region, and had probably already adapted to live in endogean or hypogean microhabitats before dispersing to the Greenbrier and Cowpasture valleys. As temperatures rose and surface conditions became drier at the end of the Pleistocene, the two lineages would have likely been driven to independently seek deeper and cooler microhabitats in the epikarst above Arbuckle Cave and Williams Cave. The geologic isolation of the two caves is absolute, as they are separated by several anticlinal ridges of insoluble rock and a distance of approximately 70 km (Fig. 13). Although subterranean organisms can surely move between human-enterable caves within the same limestone deposits, they are probably less likely to move from one limestone "island" to another. Hydrochory, transport by water, has been proposed as one mechanism by which such movement could occur during high flood conditions (Barr and Peck 1965). However, hydrochory between Arbuckle and Williams Cave is currently impossible, since the two lie within entirely different watersheds on opposite sides of the eastern Continental Divide: water in the New River watershed (Arbuckle Cave) flows to the Gulf of Mexico and water in the James River watershed (Williams Cave) flows to the Atlantic Ocean. No sister taxa that would lend support for a connection between Arbuckle and Williams Cave are known. It is possible that endogean or hypogean populations of *Horologion* still exist at high elevations in the non-karst mountains between the two caves. With the exception of trapping by Harden and colleagues near Maxwelton, WV and several sites near Williams Cave, these shallow subterranean habitats have not been directly sampled. Extensive shale deposits occur on many of the higher forested mountain slopes, and could provide a suitable cave-like microhabitat for *Horologion* and other subterranean trechines to still exist. One species of the troglobitic genus *Pseudanophthalmus* is found in shallow subterranean habitats nearby at Cranberry Glades in Pocahontas County, WV (Barr 1967), and members of the genus have also been collected from an abandoned coal mine in non-karst terrain in eastern Kentucky (Barr 1986), indicating that suitable microhabitats for "cave beetles" still exist in non-cave habitats throughout the middle Appalachians.

At a broader geographic and taxonomic scale, the combined evidence from molecular and morphological data suggests that the most likely sister to *Horologion* is the tribe Bembidarenini, which occurs only in the southern hemisphere, making *Horologion* a true relict of a formerly widespread clade, and an important component of Appalachian biodiversity. While characterizing the species of *Horologion* as "single cave endemics" (Christman et al. 2005) is perhaps not accurate if the caves are not the true habitat, the species are certainly worthy of conservation. Other examples of isolated relicts that have apparently survived by adapting to live in caves are known within Carabidae. For example, *Dalyat mirabilis* Mateu is the only Palearctic representative of the subfamily Promecognathinae, which is otherwise known from the Pacific Northwest of North America and South Africa (Mateu and Bellés 2003; Ribera et al. 2005).

Conclusions

Our reexamination of the male type of *H. speokoites* and detailed examination of males and females of *H. hubbardi* allow us to confidently place *Horologion* in the supertribe Trechitae, in a clade that also contains the tribes Bembidarenini and Trechini. The combined morphological and molecular data strongly support a relationship with Trechini and the Gondwanan tribe Bembidarenini; the molecular data provide moderate support for a sister relationship with Bembidarenini. More extensive taxon sampling for molecular analyses including lovriciines could help solidify the systematic position of *Horologion*, as could more extensive DNA and morphological data of trechites.

The two species of *Horologion* represent a unique lineage within the Appalachian region and the entire northern hemisphere. Additional populations and species of *Horologion* probably remain to be discovered. That such elusive but distinctive taxa can be discovered in well-trodden areas highlights our limited knowledge of global biodiversity, and encourages experimentation with more diverse approaches to document it fully.

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Supplementary material 1

PCR primers and protocols

Authors: Curt W. Harden, Robert L. Davidson, Thomas E. Malabad, Michael S. Caterino, David R. Maddison

Data type: pdf

Explanation note: **table S1.** List of primers used in amplification ($_a$) and Sanger sequencing ($_b$); **table S2.** PCR programs. All began with an initial denaturing phase of 180s at 94 °C, with each cycling phase; **table S3.** PCR protocols for gene fragments. For nested and hemi-nested reactions, programs and primers for inner and outer reactions are indicated by [1] and [2], respectively.

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Link: https://doi.org/10.3897/subtbiol.48.114404.suppl1

Supplementary material 2

Taxa sampled for phylogenetic analyses

Authors: Curt W. Harden, Robert L. Davidson, Thomas E. Malabad, Michael S. Caterino, David R. Maddison

Data type: pdf

Explanation note: **table S4.** table of taxa sampled for phylogenetic analyses, with associated GenBank numbers.

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Supplementary material 3

Additional photos of *Horologion* and Bembidarenini

Authors: Curt W. Harde, Robert L. Davidson, Thomas E. Malabad, Michael S. Caterino, David R. Maddison

Data type: pdf

Explanation note: **figure S1.** Habitus of *Horologion hubbardi*; **figure S2.** *Horologion speokoites* holotype (USNMENT 01374911); **figure S3.** *Horologion hubbardi*, SEM photographs of individual found dead (VMNH); **figure S4.** SEM photographs of the ligula, ventral aspect.

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Supplementary material 4

Trees from Maximum Likelihood and Bootstrap analyses of single gene and 8-gene matrices

Authors: Curt W. Harden, Robert L. Davidson, Thomas E. Malabad, Michael S. Caterino, David R. Maddison

Data type: pdf

Explanation note: **figure S5.** Maximum likelihood trees of 8-gene concatenated matrix; **figure S6.** Maximum likelihood trees of 18S; **figure S7.** Maximum likelihood trees of 28S; **figure S8.** Maximum likelihood trees of COI; **figure S9.** Maximum likelihood trees of CAD2; **figure S10.** Maximum likelihood trees of CAD4; **figure S11.** Maximum likelihood trees of Wg; **figure S12.** Maximum likelihood trees of Topo; **figure S13.** Maximum likelihood trees of MSP; **figure S14.** Maximum likelihood trees of ArgK.

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RESEARCH ARTICLE

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DNA taxonomy reveals high species diversity among the stygobiont genus *Metastenasellus* (Crustacea, Isopoda) in African groundwater

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Abstract

This study aimed to explore the species diversity within the isopod genus *Metastenasellus* in Benin and Cameroon. Compared to other parts of the world, the described diversity of stygobiotic crustaceans in Africa is low due to a dearth of studies and taxonomic expertise. However, recent research activities in Benin and Cameroon suggest higher groundwater diversity than previously envisioned. Recent sampling campaigns in these countries have shown that *Metastenasellus* is a major group in the underground aquatic environment. The accumulation of biological material provided an opportunity to explore species diversity within the genus using a DNA taxonomy approach based on the cytochrome *c* oxidase subunit I (COI) gene fragment.

Despite the limitations of using a single-locus approach for species delimitation, an overview of the diversity within the genus *Metastenasellus* was obtained, revealing the presence of 23 distinct lineages. Several elements suggest that most, if not all, of these lineages represent valid species. These include high genetic distances between lineages, morphologically distinct species separated by genetic distances of the same order of magnitude as between other described lineages, and the coexistence of different lineages at the same stations.

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Despite a limited sampling effort, these first results indicate a high level of species diversity and endemism within *Metastenasellus* in the studied regions. The narrow geographic distribution of the lineages suggests strong isolation and limited dispersal abilities. This study highlights the potential for discovering a significant number of new species within this genus and emphasizes the need for further research to uncover the extent of diversity in African stygobiotic isopods.

Keywords
Africa, Diversity, DNA taxonomy, Endemism, Genus *Metastenasellus*, Groundwater Isopoda

Introduction

Obligate groundwater organisms, or stygobites, are known in all animal groups, including invertebrates and vertebrates (Botosaneanu 1986; Gunn 2004), but crustaceans constitute 43% of their total known diversity (Gibert and Culver 2009). In Africa, the Isopoda is the order that contains the largest number of stygobiotic species among the crustaceans (6 families, 23 genera, 80 species) (Tuekam Kayo et al. 2012). The asellote Stenasellidae is a major group in the groundwater (Magniez 1999) and is the most diverse family in Africa (24 species validly described to date), found mainly in tropical Africa, from Côte d'Ivoire to Kenya (Tuekam Kayo et al. 2012; Pountougnigni et al. 2021). Within the family, the genus *Metastenasellus* Magniez, 1966 includes nine species described to date with trans-Saharan distribution, with one species known from Algeria and 8 others distributed in West and Central Africa (DRC, Cameroon, Côte d'Ivoire, Nigeria) (Pountougnigni et al. 2021).

Compared to other parts of the world, information on the diversity and endemism of stygobiotic crustaceans found in Africa remain very low due to a lack of studies and a deficit in taxonomic expertise (Boutin et al. 2011; Tuekam Kayo et al. 2012). Recent years have seen the development of research activities in both Benin and Cameroon to document groundwater biodiversity in relation to water quality, vulnerability to pollution and local use (see among others Tuekam Kayo 2013; Lagnika et al. 2014; Lagnika 2015; Martin et al. 2019; Tuekam Kayo et al. 2021). First results suggest a much higher stygofaunal diversity in these countries than is currently known. The stygobiotic genus *Metastenasellus* is a good biological model to investigate this issue.

Although no species has yet been formally described in Benin, *Metastenasellus* has been observed on several occasions, during surveys of the faunistic and water quality of wells in that country (Lagnika et al. 2014; Lagnika 2015). Sequences of the cytochrome *c* oxidase subunit (COI) gene fragment are available for two *Metastenasellus* specimens from Benin (Eme et al. 2018). In Cameroon, Zébazé Togouet et al. (2009) reported for the first time two undescribed *Metastenasellus* species in the country. The genus was mentioned in several wells of Yaoundé (Tuekam Kayo 2013) and *Metastenasellus camerounensis* Zébazé Togouet, Boulanouar, Njiné & Boutin, 2013 was eventually the first species to be described in Cameroon (Zébazé Togouet et al. 2013). Later, two undescribed species were mentioned from the Bamoun Plateau in the Western Region of

Cameroon (Nana Nkemegni et al. 2015). Although initially reported as *Stenasellus* species, these two species were later treated as species of *Metastenasellus* (Pountougnigni et al. 2021), which is indeed more consistent with the disjunct distribution of these genera in Africa. First COI sequences were provided for *Metastenasellus* specimens from four stations in Cameroon (Eme et al. 2018). *M. camerounensis* was identified in the two closest stations, while the other two stations, about 70 km geographically distant from each other, proved to harbour two distinct, as yet undescribed species. Recently, a second species, *M. boutini* Poutougnigni, Piscart & Zebaze Togouet, 2021 was described from Douala city. Thus, although knowledge of the stygofauna is still in its infancy in West and Central Africa, the first results show that *Metastenasellus* is indeed present in Benin and Cameroon, and that its species diversity may be much higher than expected.

In the last few years, several stygofauna sampling campaigns in hand-dug wells have taken place in Benin and in Cameroon. They made it possible to accumulate biological material of *Metastenasellus* in dozens of wells in both countries. This material gives us the opportunity to provide a first insight into the species diversity within the genus *Metastenasellus* in these two countries, using a Sanger-based DNA barcoding approach (Hebert et al. 2003).

Materials and methods

Specimens

In Benin, a series of sampling campaigns was organized between 2015 and 2019 in the large Ouémé watershed. A collection of isopod specimens was obtained from a sampling of 169 stations in the Ouémé/ Yéwa basin, 98 of which contained isopods (Fig. 1). Samples were taken in traditional hand-dug, as well as in modern wells lined with casing (as described in BURGÉAP 1981) by means of a modified Cvetkov phreatobiological net (funnel 200 µm mesh size, 150 µm below valve) (Cvetkov 1968; Boutin et al. 2011). For each sampled well, traps baited with a piece of beef were used to collect bottom dwelling animals (Lagnika et al. 2014). Traps were deposited at the bottom of the well for about 24 hours (depending on field constraints). Faunal samples were fixed in ethanol 95% on the spot and were later sorted out at the laboratory of the Zoology Department of the Abomey-Calavi University. Isopod specimens from the same sample were stored in the same vial, preserved in 95% ethanol, and kept in the fridge at 5 °C. Samples were later transferred to the Royal Belgian Institute of Natural Sciences for further processing and were kept at -20 °C in a deep freezer.

In Cameroon, a total of about 150 stations were sampled during field campaigns organized during 2009 and 2019 (Fig. 3). The collection of fauna from the wells was done following the same protocol as in Benin (modified phreatobiological net and baited traps). Baited traps were in place for a period of 10 to 18 h, following the recommendations of Boutin and Boulanouar (1983). The stations were distributed in three major watersheds, namely the Wouri (northwestern Coastal rivers basin), the Sanaga

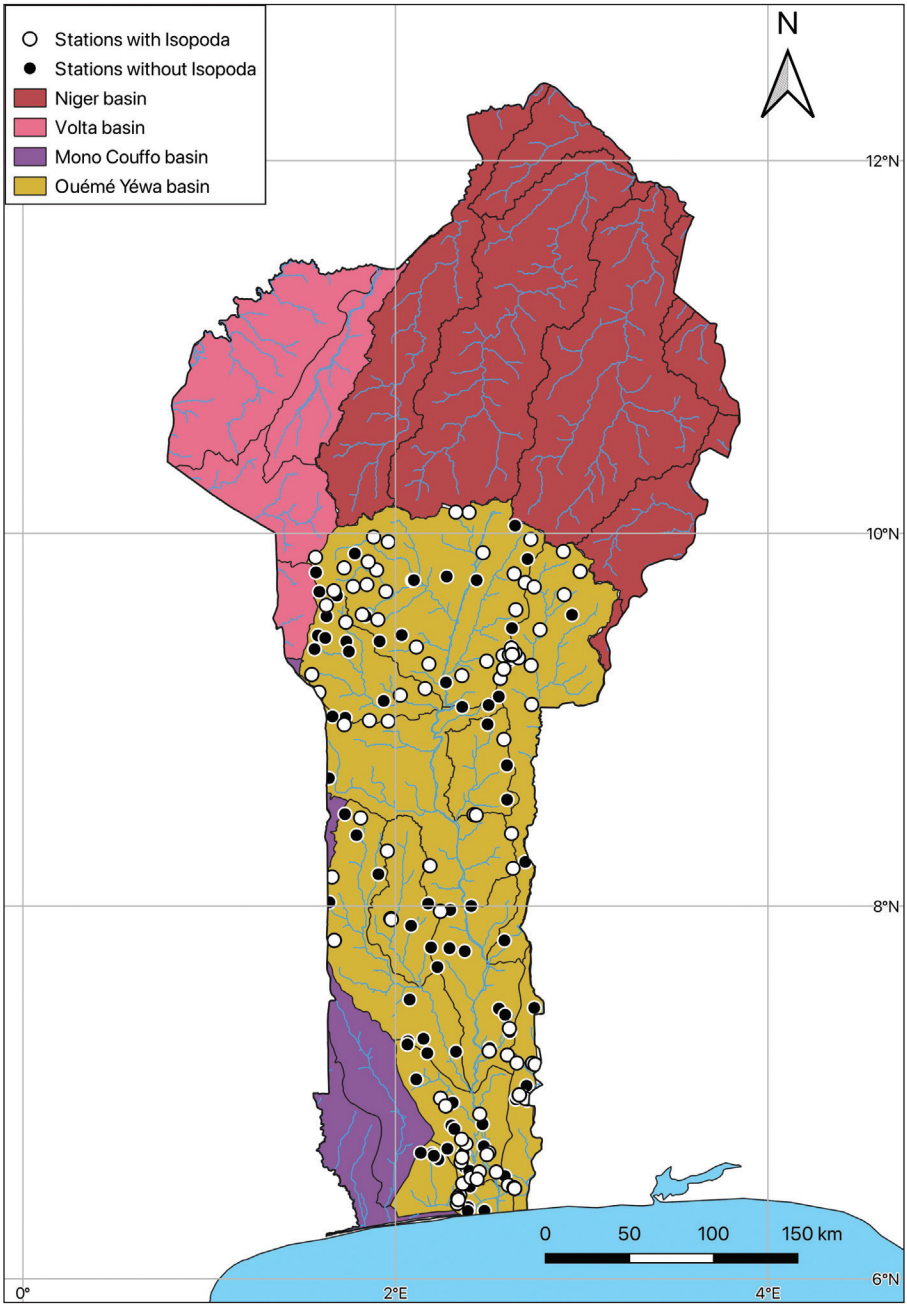

Figure 1. Location of stations sampled in Benin between 2015 and 2019.

and the Nyong (southern Coastal rivers basin). Specimens of the genus *Metastenasellus* were found at 44 stations (Fig. 4), preserved in 95% ethanol, and stored in a freezer at – 4 °C for further molecular processing.

DNA analyses

Specimens

We analyzed a dataset consisting of 89 *Metastenasellus* specimens, of which 57 were collected in Benin, and 32 in Cameroon. One Stenasellidae, *Stenasellus virei*, was used as an outgroup for tree rooting (see below) (Table 1).

The identification of the genus *Metastenasellus* was based on the Magniez (1966) diagnosis, later emended by Magniez (1979) and Zébazé Togouet et al. (2013), and confirmed by Pountougnigni et al. (2021): pleonites 1 and 2 well-developed, dactyli of pereopods 2–7 with one sternal spine, male protopodite of pleopod 1 without a coupling hook, male endopodite of pleopod 2 very voluminous, helicoidal spermatic duct, length ratio of pleonites 1 and 2 to pereonite 7 equal to 2/3 to 1/2.

For Benin, specimens came from a selection of 19 stations chosen so as to have a balanced geographical distribution within the Ouémé basin (Fig. 2). A maximum of three specimens were processed per sample, giving priority to males as their pleopods offer good taxonomic characters for the recognition and further description of new species (Magniez 1966; Magniez and Henry 1983). DNA was extracted from two pereopods, where possible. Specimens were immersed in glycerin for 12 to 24 hours before dissection to soften the appendages made brittle by prolonged stay in ethanol. They were then placed back in 95° ethanol after passing through successive ethanol baths of increasing concentrations. Regarding Cameroon, DNA was extracted and purified from one or two legs (pereopod 1) for large organisms, whereas whole individual were sometimes used for smaller specimens.

DNA extraction and sequencing

DNA was extracted and purified using the Nucleospin Tissue Kit (Macherey-Nagel). Amplification of the COI marker was done by polymerase chain reaction (PCR) followed by Sanger sequencing, using, for the Beninese material, the primers of Folmer et al. (1994) (LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3'; HC02198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3') and, for the Cameroonian material, their slightly modified version by Astrin and Stüben (2008) (LCO1490-JJ": 5'-CHACWAAYCATAAAGATATYGG-3'; HCO2198-JJ: 5'-AWACTTCVGGRTGVCCAAARAATCA-3'). The PCR mixtures (total volumes of 20 and 50 µl for samples from Benin and Cameroon, respectively) consisted of 1 and 8 µl of DNA extract, final concentrations of 0.4 and 0.16 µM of each primer, 0.03 and 0.025 units/µl of Platinum Taq and DreamTaq DNA Polymerase, 1× reaction buffer, 0.2 mM of dNTP and 1.5 mM of MgCl. The samples were then run in a PCR Thermal Cycler with the following program: an initial denaturation of 3 min at 94 °C followed by 40 and 36 cycles for samples from Benin and Cameroon, respectively of 20 s at 94 °C, 45 s at 50 °C and 60 s at 72 °C and 65 °C. A final extension step of 5 and 2 min at 72 °C and 65 °C was performed in Benin and

Table 1. Specimens included in the study, with their place of deposit (POD – RBINS: Royal Belgian Institute of Natural Sciences; ULB: "Université libre de Bruxelles"), voucher numbers, isolate, MOTUs identified in the ASAP and GMYC analyses, collection data (country, region/department, locality, municipality, latitude, longitude –datum WGS84, station, collection date, collector) and GenBank accession numbers.

Species	POD	Voucher	Isolate	MOTUs	Country	Region/ Department	Municipality; locality	Y coord.	X coord.	Station ID	Collection date	Collector	COI GenBank
Metastenasellus sp.	RBINS	19.304.08	LM423	1	Benin	Plateau	Pobè	7.14983	2.75058	IBT	24-09-2019	M. Lagnika et al	OR797545
	RBINS	19.311.04	LM444	1	Benin	Plateau	Pobè	7.14983	2.75058	IBT	24-09-2019	M. Lagnika et al	OR797559
	RBINS	19.301.10	LM401	2	Benin	Borgou	Ina-CLCAM	9.96883	2.72742	BEN106	1-08-2017	M. Lagnika et al	OR797526
	RBINS	19.301.11	LM402	2	Benin	Borgou	Ina-CLCAM	9.96883	2.72742	BEN106	1-08-2017	M. Lagnika et al	OR797527
	RBINS	19.301.12	LM403	2	Benin	Borgou	Ina-CLCAM	9.96883	2.72742	BEN106	1-08-2017	M. Lagnika et al	OR797528
	RBINS	19.302.02	LM405	2	Benin	Borgou	Gnanhou	9.90350	2.90209	BEN108	2-08-2017	M. Lagnika et al	OR797529
	RBINS	19.302.07	LM410	2	Benin	Borgou	Woria	9.08218	2.73199	BEN143	26-08-2017	M. Lagnika et al	OR797533
	RBINS	19.302.08	LM411	2	Benin	Borgou	Woria	9.08218	2.73199	BEN143	26-08-2017	M. Lagnika et al	OR797534
	RBINS	19.302.09	LM412	2	Benin	Borgou	Woria	9.08218	2.73199	BEN143	26-08-2017	M. Lagnika et al	OR797535
	RBINS	19.302.10	LM413	2	Benin	Borgou	Tchori	9.67146	2.90645	BEN144	27-08-2017	M. Lagnika et al	OR797536
	RBINS	19.302.11	LM414	2	Benin	Borgou	Tchori	9.67146	2.90645	BEN144	27-08-2017	M. Lagnika et al	OR797537
	RBINS	19.302.12	LM415	2	Benin	Borgou	Tchori	9.67146	2.90645	BEN144	27-08-2017	M. Lagnika et al	OR797538
	RBINS	19.311.02	LM442	2	Benin	Borgou	Ina-CLCAM	9.96883	2.72742	BEN106	1-08-2017	M. Lagnika et al	OR797557
	RBINS	19.311.03	LM443	2	Benin	Borgou	Gnanhou	9.90350	2.90209	BEN108	2-08-2017	M. Lagnika et al	OR797558
	RBINS	19.308.11	LM438	3	Benin	Borgou	Parakou	9.35047	2.62711	C6	21-07-2018	M. Lagnika et al	OR797554
	RBINS	19.308.12	LM439	3	Benin	Borgou	Parakou	9.35047	2.62711	C6	21-07-2018	M. Lagnika et al	OR797555
	RBINS	19.311.06	LM446	3	Benin	Borgou	Parakou	9.38486	2.62311	C2	22-09-2019	M. Lagnika et al	OR797560
	RBINS	19.311.07	LM447	3	Benin	Borgou	Parakou	9.35047	2.62711	C6	21-07-2018	M. Lagnika et al	OR797561
	RBINS	19.311.08	LM448	3	Benin	Borgou	Parakou	9.35047	2.62711	C6	21-07-2018	M. Lagnika et al	OR797562
	RBINS	19.311.09	LM449	3	Benin	Borgou	Parakou	9.35047	2.62711	C6	21-07-2018	M. Lagnika et al	OR797563
	RBINS	19.323.02	LM451	3	Benin	Borgou	Parakou	9.35047	2.62711	C6	27-07-2017	M. Lagnika et al	OR797564
	RBINS	19.323.03	LM452	3	Benin	Borgou	Parakou	9.35047	2.62711	C6	27-07-2017	M. Lagnika et al	OR797565
	RBINS	19.304.12	LM427	4	Benin	Plateau	Pobè	6.98711	2.66572	ISH	26-09-2019	M. Lagnika et al	OR797547
	RBINS	19.297.11	LM390	5	Benin	Collines	Agoua N'Tchotche	8.29609	1.95661	BEN052	25-07-2016	M. Lagnika et al	OR797519
	RBINS	19.323.08	LM457	6	Benin	Collines	Tchètti Lema	7.81608	1.67185	BEN072	10-08-2016	M. Lagnika et al	OR797570
	RBINS	19.323.10	LM459	6	Benin	Collines	Tchètti Lema	7.81608	1.67185	BEN072	31-07-2017	M. Lagnika et al	OR797572
	RBINS	19.304.04	LM419	7	Benin	Collines	Tchètti Lema	7.81608	1.67185	BEN072	15-08-2018	M. Lagnika et al	OR797542
	RBINS	19.304.05	LM420	7	Benin	Collines	Tchètti Lema	7.81608	1.67185	BEN072	15-08-2018	M. Lagnika et al	OR797543
	RBINS	19.323.09	LM458	7	Benin	Collines	Tchètti Lema	7.81608	1.67185	BEN072	10-08-2016	M. Lagnika et al	OR797571
	RBINS	19.323.11	LM460	7	Benin	Collines	Tchètti Lema	7.81608	1.67185	BEN072	31-07-2017	M. Lagnika et al	OR797573

Species	POD	Voucher	Isolate	MOTUs	Country	Region/ Department	Municipality; locality	Y coord.	X coord.	Station ID	Collection date	Collector	COI GenBank
Metastenasellus sp.	RBINS	19.301.04	LM395	8	Benin	Donga	Manigri Idiroko	8.97372	1.72553	BEN079	15-08-2016	M. Lagnika et al	OR797522
	RBINS	19.301.07	LM398	8	Benin	Donga	Penessoulou Cs	9.24313	1.55093	BEN086	16-08-2016	M. Lagnika et al	OR797523
	RBINS	19.301.08	LM399	8	Benin	Donga	Penessoulou Cs	9.24313	1.55093	BEN086	16-08-2016	M. Lagnika et al	OR797524
	RBINS	19.301.09	LM400	8	Benin	Donga	Penessoulou Cs	9.24313	1.55093	BEN086	16-08-2016	M. Lagnika et al	OR797525
	RBINS	19.302.04	LM407	8	Benin	Donga	Gangamou	9.84855	1.85416	BEN114	5-08-2017	M. Lagnika et al	OR797530
	RBINS	19.302.05	LM408	8	Benin	Donga	Gangamou	9.84855	1.85416	BEN114	5-08-2017	M. Lagnika et al	OR797531
	RBINS	19.302.06	LM409	8	Benin	Donga	Gangamou	9.84855	1.85416	BEN114	5-08-2017	M. Lagnika et al	OR797532
	RBINS	19.301.01	LM392	9	Benin	Collines	Ouessé CSC	8.48835	2.43420	BEN059	28-07-2016	M. Lagnika et al	OR797520
	RBINS	19.301.03	LM394	9	Benin	Collines	Ouessé CSC	8.48835	2.43420	BEN059	28-07-2016	M. Lagnika et al	OR797521
	RBINS	19.323.04	LM453	9	Benin	Plateau	Oke-Ola	7.22220	2.50389	BEN031	2-08-2015	M. Lagnika et al	OR797566
	RBINS	19.308.05	LM432	10	Benin	Ouémé	Porto-Novo	6.48492	2.64036	LB	29-06-2019	M. Lagnika et al	OR797551
	RBINS	19.308.06	LM433	10	Benin	Ouémé	Porto-Novo	6.48492	2.64036	LB	29-06-2019	M. Lagnika et al	OR797552
	RBINS	19.308.08	LM435	10	Benin	Ouémé	Porto-Novo	6.48856	2.63744	AH	29-09-2019	M. Lagnika et al	OR797553
	RBINS	19.309.13	LM440	10	Benin	Ouémé	Porto-Novo	6.48450	2.64008	AR	23-06-2019	M. Lagnika et al	OR797556
				10	Benin	Ouémé	Porto-Novo	6.48492	2.64036	LB		M. Lagnika et al	KY623773
				10	Benin	Ouémé	Porto-Novo	6.48492	2.64036	LB		M. Lagnika et al	KY623774
	RBINS	19.304.01	LM416	11	Benin	Plateau	Oke-Ola	7.22220	2.50389	BEN031	9-06-2018	M. Lagnika et al	OR797539
	RBINS	19.304.02	LM417	11	Benin	Plateau	Oke-Ola	7.22220	2.50389	BEN031	9-06-2018	M. Lagnika et al	OR797540
	RBINS	19.304.03	LM418	11	Benin	Plateau	Oke-Ola	7.22220	2.50389	BEN031	9-06-2018	M. Lagnika et al	OR797541
	RBINS	19.304.07	LM422	11	Benin	Plateau	Pobè	7.14983	2.75058	IBT	24-09-2019	M. Lagnika et al	OR797544
	RBINS	19.304.09	LM424	11	Benin	Plateau	Pobè	7.14983	2.75058	IBT	24-09-2019	M. Lagnika et al	OR797546
	RBINS	19.308.01	LM428	11	Benin	Plateau	Pobè	7.15653	2.73597	TW	24-09-2019	M. Lagnika et al	OR797548
	RBINS	19.308.03	LM430	11	Benin	Plateau	Pobè	7.15653	2.73597	TW	24-09-2019	M. Lagnika et al	OR797549
	RBINS	19.308.04	LM431	11	Benin	Plateau	Pobè	7.15653	2.73597	TW	24-09-2019	M. Lagnika et al	OR797550
	RBINS	19.323.05	LM454	11	Benin	Plateau	Oke-Ola	7.22220	2.50389	BEN031	27-01-2017	M. Lagnika et al	OR797567
	RBINS	19.323.06	LM455	11	Benin	Plateau	Oke-Ola	7.22220	2.50389	BEN031	27-01-2017	M. Lagnika et al	OR797568
	RBINS	19.323.07	LM456	11	Benin	Plateau	Oke-Ola	7.22220	2.50389	BEN031	27-01-2017	M. Lagnika et al	OR797569
	ULB	RK19_14	RK19_14	12	Cameroon	Littoral	Mbanga; Mb1	4.29030	9.34022		11-2018	R. Kayo	OR791025
	ULB	RK19_01	RK19_01	12	Cameroon	Littoral	Mbanga; Mb1	4.29030	9.34022		10-2018	R. Kayo	OR791023
	ULB	RK19_88	RK19_88	12	Cameroon	Southwest	Muyuka; Owé 3S	4.17264	9.23050		11-2017	R. Kayo & Chinche	OR791041
				12	Cameroon	Littoral	Moungo; Loum 1	4.43100	9.43300				KY623775
	ULB	RK19_34	RK19_34	13	Cameroon	West	Foumbam; Fbt6	5.29550	10.3718		11-2018	R. Kayo & Farikou	OR791036
				14	Cameroon	Littoral	Douala; Makepe 2	4.06800	9.72100				KY623776

Species	POD	Voucher	Isolate	MOTUs	Country	Region/ Department	Municipality; locality	Y coord.	X coord.	Station ID	Collection date	Collector	COI GenBank
Metastenasellus				15	Cameroon	Littoral	Douala; P1	4.12411	9.82686				OL514108
boutini				15	Cameroon	Littoral	Douala; P3	4.12144	9.82833				OL514109
				15	Cameroon	Littoral	Douala; P4	4.12111	9.82817				OL514110
				15	Cameroon	Littoral	Douala; P10	4.11908	9.82592				OL514111
				15	Cameroon	Littoral	Douala; P3	4.12144	9.82833				OL514112
Metastenasellus sp.	ULB	RK19_15	RK19_15	16	Cameroon	Central	Nkoteng; Addic	4.43160	12.09130		11-2018	R. Kayo & Mdejo	OR791026
	ULB	RK19_19	RK19_19	17	Cameroon	Southwest	Muyuka; OS2	4.17542	9.22399		11-2017	R. Kayo & Chinche	OR791030
	ULB	RK19_18	RK19_18	18	Cameroon	Southwest	Tiko; TW10	4.04531	9.22081		02-2017	R. Kayo & Chinche	OR791029
	ULB	RK19_32	RK19_32	18	Cameroon	Southwest	Tiko; TW3	4.04543	9.21073		08-2017	R. Kayo & Chinche	OR791035
	ULB	RK19_23	RK19_23	19	Cameroon	West	Dcshang; Pb7C3	5.27080	10.04340		03-2018	R. Kayo & Madiesse	OR791032
	ULB	RK19_17	RK19_17	20	Cameroon	West	Dschang; PP5C2MKN	5.56420	10.31230		03-2018	R. Kayo & Madiesse	OR791028
	ULB	RK19_25	RK19_25	21	Cameroon	West	Dschang; Pb6C2MKN	5.56060	10.04670		02-2018	R. Kayo & Madiesse	OR791033
	ULB	RK19_30	RK19_30	21	Cameroon	West	Dschang; Pd9C1MKN	5.34650	10.08620		04-2018	R. Kayo & Madiesse	OR791034
	ULB	RK19_37	RK19_37	21	Cameroon	West	Dschang; Pd6C1	5.42670	10.07910			R. Kayo & Madiesse	OR791038
	ULB	RK19_22	RK19_22	22	Cameroon	Central	Nkoteng; Essoboutou	4.33150	12.08140		08-2016	R. Kayo & Mdejo	OR791031
	ULB	RK19_35	RK19_35	22	Cameroon	Central	Nkoteng; Camp Nouveau	4.45120	12.08190		10-2016	R. Kayo & Mdejo	OR791037
	ULB	RK19_02	RK19_02	23	Cameroon	Central	Yaoundé; Olembé	3.41247	11.26854		06-2017	R. Kayo & Tayou	OR791024
	ULB	RK19_16	RK19_16	23	Cameroon	Central	Olembé; Olembé3	3.49720	11.33966		11-2018	R. Kayo & Tayou	OR791027
	ULB	RK19_47	RK19_47	23	Cameroon	Central	Yaoundé; PM3	3.55212	11.24516		08-2017	R. Kayo & Tayou	OR791039
	ULB	RK19_48	RK19_48	23	Cameroon	Central	Yaoundé; PM5	3.50010	11.26853		06-2017	R. Kayo & Tayou	OR791040
	ULB	RK19_93	RK19_93	23	Cameroon	Central	Yaoundé; Olembé III	3.49720	11.33966		05-2017	R. Kayo & Tayou	OR791042
	ULB	RK19_95	RK19_95	23	Cameroon	Central	Yaoundé; Messassi 1	3.41612	11.26231		05-2017	R. Kayo & Tayou	OR791043
Metastenasellus				23	Cameroon	Central	Yaoundé; Emana	3.91900	11.52000				KY623769
camerounensis				23	Cameroon	Central	Mefou-et-Akono; Ebogo 1	3.56800	11.35400				KY623772
				23	Cameroon	Central	Yaoundé; Emana	3.91900	11.52000				KY623770
				23	Cameroon	Central	Yaoundé; Emana	3.91900	11.52000				KY623771
Stenasellus virei					Spain	Guadalajara	Trillo	40.69067	-2.58333				JQ921669

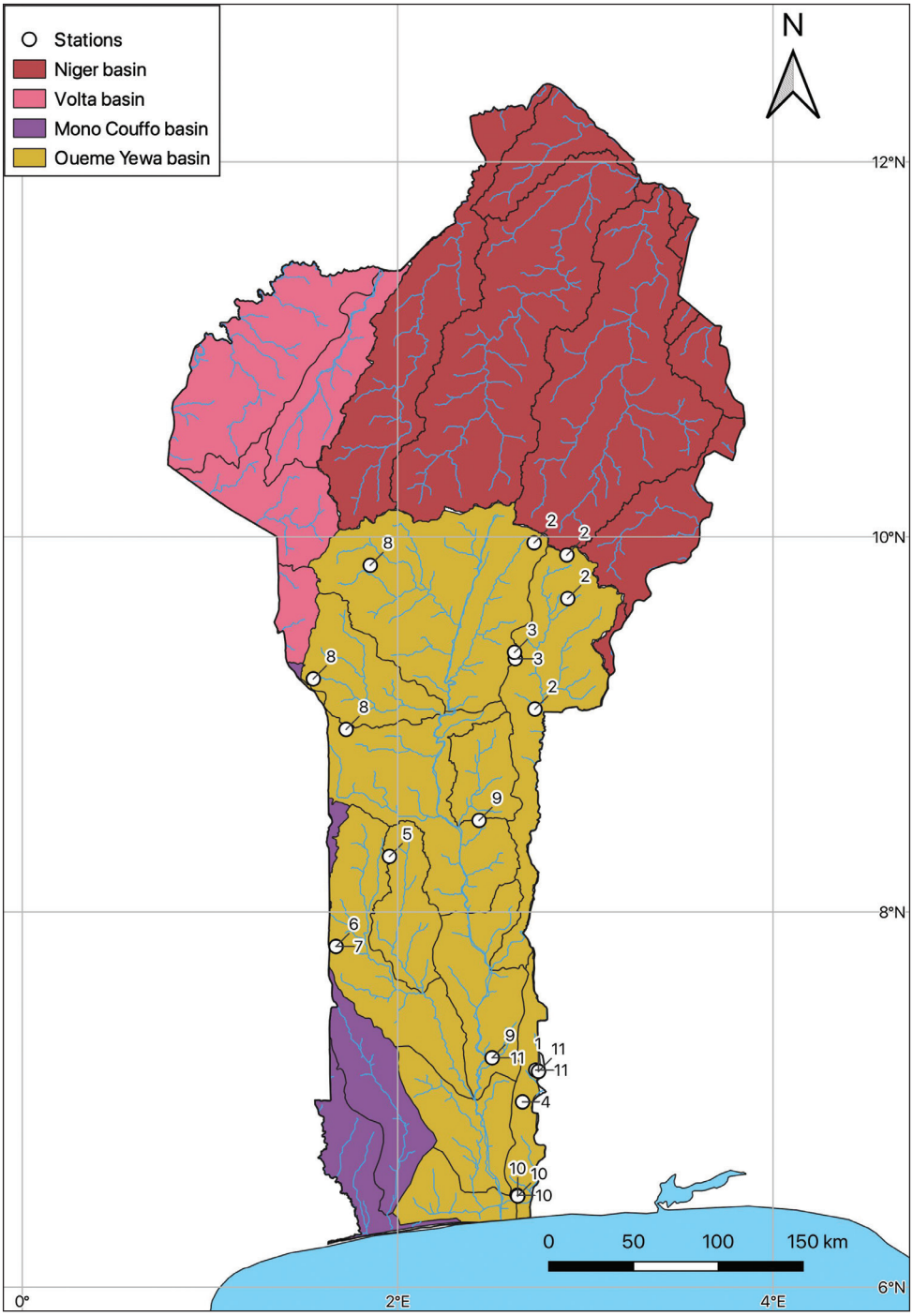

Figure 2. Location of the 19 stations in the Ouémé basin (Benin) from which 57 *Metastenasellus* specimens were selected for DNA taxonomy. The numbers connected to the station symbols correspond to the numbering of the different MOTUs identified in this study.

Cameroon, respectively. PCR products were checked after migration by electrophoresis in a 1.2% agarose gel and, for Benin, were purified using the ExoSAP procedure (Exonuclease I– Shrimp Alkaline Phosphatase from ThermoFisher, USA). Successful amplicons were sequenced bidirectionally at Macrogen Europe BV (Amsterdam, The Netherlands) and at Laboratoire Genoscreen (France) for samples from Benin and Cameroon, respectively.

The resulting sequences were assembled and cleaned using CodonCode Aligner v.8.0.2 (CodonCode Corporation) and Sequencher v.4.1.4 (Gene Codes Corporation), and compared to sequences already published in international databases using NCBI BLAST (NCBI Resource Coordinators 2016). For Benin, 76 contigs were obtained from the 81 DNA extracts and, after elimination of poor-quality sequences, a final selection of 57 contigs (specimens from 19 different stations) was retained. This dataset was supplemented by 2 additional sequences of *Metastenasellus* made available on GenBank by Eme et al. (2018), from specimens collected in Benin by one of us (ML).

For Cameroon, the 29 contigs obtained in this study were completed by 4 sequences made available by Eme et al. (2018) in GenBank, from specimens collected by one of us (RTK), as well as 5 sequences of *M. boutini* published by Pountougnigni et al. (2021) (Table 1).

Molecular phylogeny

A phylogenetic tree was inferred by maximum likelihood using IQ-TREE v. 2.2.0 for macOS (Nguyen et al. 2015), with the best-fit model, TN+F+I+G4, automatically selected by the software, according to the Bayesian Information Criterion, via ModelFinder (Kalyaanamoorthy et al. 2017), as well as optimisation of its parameters, and data partitioned according to codon position. Branch support was obtained with the ultrafast bootstrap with 1000 replicates (Hoang et al. 2018).

Distance analysis

Uncorrected pairwise genetic distances were calculated using MEGA 11 (Tamura et al. 2021), after trivial alignment of COI sequences facilitated using the MUSCLE algorithm (Edgar 2004). Genetic distances were calculated between sequences, and between and within MOTUs (Molecular Operational Taxonomic Units) as identified by the single-locus approaches ASAP and GMYC (see below).

Single-locus species delimitation

Species were delineated following complementary approaches (Dellicour and Flot 2015): a distance-based method, ASAP ("Assembling Species by Automatic Partitioning") (Puillandre et al. 2021), and two tree-based methods, the "General Mixed Yule Coalescent" models, GMYC (Pons et al. 2006), and the "Poisson Tree Processes" (PTP) method (Zhang et al. 2013).

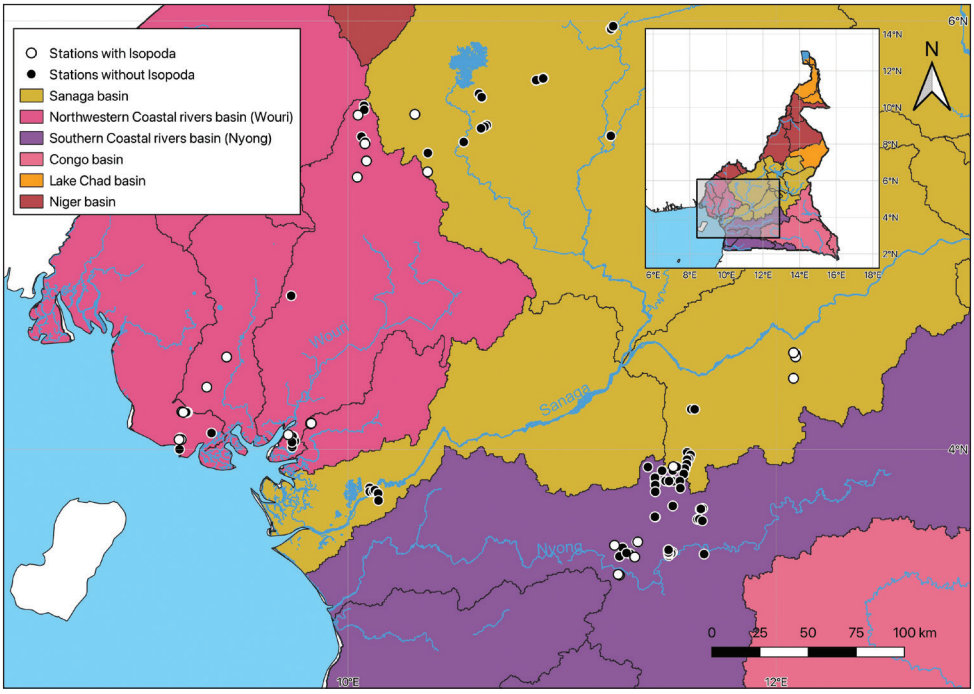

Figure 3. Location of stations sampled in Cameroon between 2009 and 2019.

ASAP was run using p-distances as well as both the Jukes-Cantor (JC69) and the Kimura 2-parameter (K80) substitution models to compute the distances, in order to investigate the possible impact of different distance models on the partitioning. Analyses were performed on the dedicated public web server (https://bioinfo.mnhn.fr/abi/public/asap/).

For the GMYC analysis, transition between inter- and intra-species branching rates were estimated on an ultrametric tree reconstructed using BEAST v2.7.2 (Bouckaert et al. 2019) (without time calibration). The Bayesian inference of phylogeny was performed using the TN+F+I nucleotide substitution model, as identified in the IQ-TREE analysis, with four gamma categories estimated by the software, a strict molecular clock model and the Yule prior with default parameters. The analysis was run with a Markov Chain Monte Carlo (MCMC) length of 10 million. The first 10% of the trees were discarded as "burn-in" and marginal posterior estimates were checked using Tracer v1.7.2. (Rambaut et al. 2021). The maximum credibility tree obtained from the BEAST analysis was imported in R v4.2.2 and submitted to the gmyc function available in the R package splits v1.0–20 (Ezard et al. 2021).

PTP analyses were performed using multi-rate PTP (mPTP), which, unlike PTP, takes into account differences in intraspecific variation, due to the evolutionary history or sampling of each species. mPTP is presented as an improvement on the single-rate model PTP (Zhang et al. 2013), making possible to obtain more accurate estimates than the latter (Kapli et al. 2017). bPTP, the Bayesian implementation of the single-rate PTP, was also used to check the congruence of results with mPTP. Unlike GMYC,

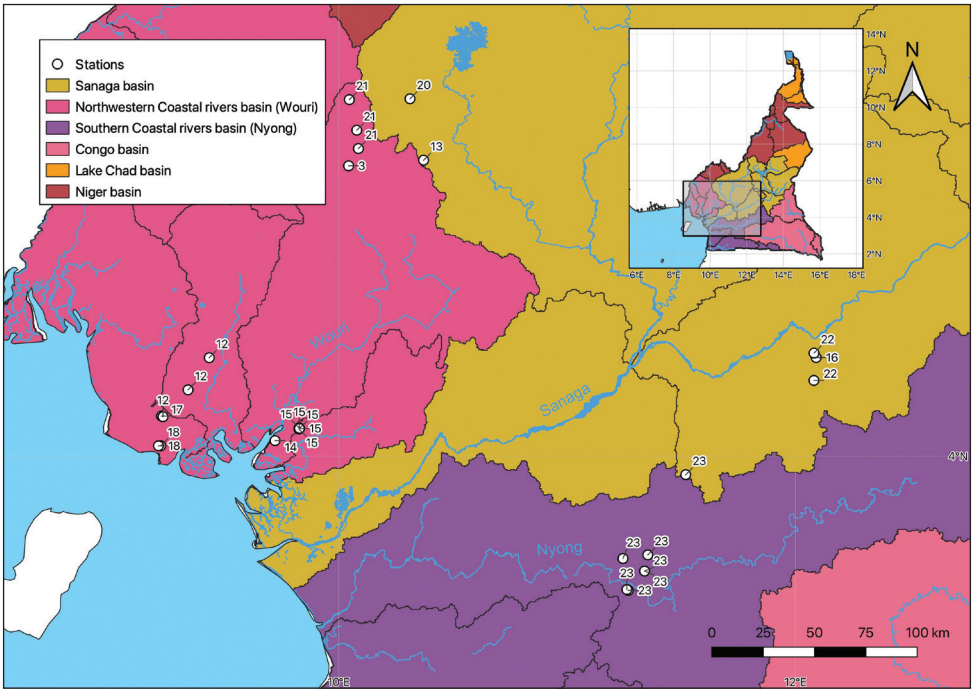

Figure 4. Location of the 44 stations where *Metastenasellus* specimens were found in Cameroon and studied for DNA taxonomy (white dots). The numbers connected to the station symbols correspond to the numbering of the different MOTUs identified in this study.

PTP analyses do not require an ultrametric input tree, which is a potentially error-prone process (Zhang et al. 2013). Therefore, the phylogenetic tree produced by the I-QTREE analysis was used as the input tree for all analyses based on the PTP model. bPTP analysis was performed on the bPTP web server (https://species.h-its.org/). The stand-alone version of mPTP was preferred to its web implementation because certain functionalities are not available in the web service (https://mptp.h-its.org/), in particular the computation of support values for each clade, using MCMC. The last release of the pre-compiled macOS binary (mPTP 0.2.4) was downloaded from GitHub (https://github.com/Pas-Kapli/mptp).

Results

Species delimitation

Both ASAP or GMYC delineated 23 similar MOTUs that corresponded to singletons or strongly supported clades in the ML tree (BV: 99–100) (Fig. 5). ASAP analyses consistently suggested the same partitioning into 23 different MOTUs, regardless of how the distances were estimated (p-distances, JC69, K80), 11 MOTUs in Benin and 12 MOTUs in Cameroon.

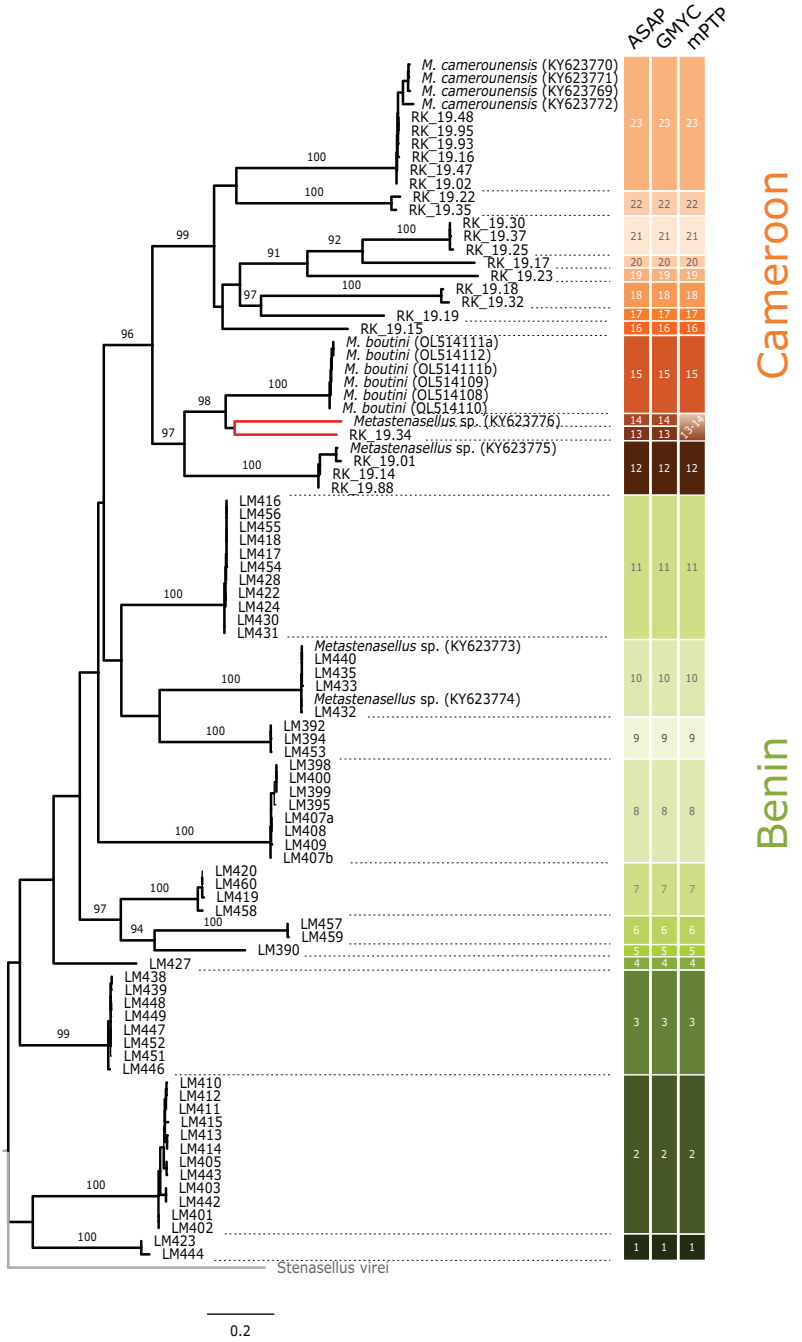

Figure 5. Molecular phylogeny constructed using the maximum likelihood method and COI gene fragment of *Metastenasellus* specimens from Benin and Cameroon. Partitions at the right side of the figure represent the results of the species delimitation analyses with single-locus methods (ASAP, GMYC, bPTP). Numbers at nodes are ultrafast bootstrap values (BV). Nodes were considered as supported if BVs were higher or equal to 90 (Hoang et al. 2018). For the sake of clarity, BVs are not shown within MOTUs delimited by ASAP and GMYC.

mPTP delineated 22 MOTUs, the same as ASAP and GMYC except for MOTUs 13 and 14 which were lumped, albeit separated by long branches on the tree (in red; Fig. 5), and uncorrected pairwise distances as high as 19.2% (Table 2; see below). However, this delimitation was the only one to receive no support (0.2), unlike all the others which received support values ranging from 0.64 to 1.00.

In contrast, bPTP delineated 26 MOTUs. Compared to others approaches, MOTU 1 was split into two singleton MOTUs, and MOTU 12 into two singleton MOTUs (RK_19.88), (RK_19.14) and a duo MOTU (KY623775, RK_19.01). Unlike mPTP, MOTUs 13 and 14 remained distinct.

Distance analysis

Uncorrected pairwise distances between specimens ranged between 0.0 and 28.6%. Considering the 23 MOTUs defined according to the results of the species delimitation analyses performed with ASAP and GMYC (Fig. 5), the maximal distances within MOTUs varied between 0.2% (MOTU 9, MOTU 10) and 3.5% (MOTU 12, MOTU 22) while the mean distances between MOTUs varied between 17.1% (M5, M7) and 27.5% (M8, M18; M9, M23; M10, M22; M12, MM18) (Table 2).

Discussion

Species delimitation

The main objective of this study was to provide a first insight into the species diversity within the genus *Metastenasellus* in Benin and Cameroon. For this, an accurate delimitation of species is not yet required, although it will be desirable in the future. In this respect, the use of a single-locus approach as a first step in a species delimitation is justified, despite its well-known weaknesses (Leliaert et al. 2014). Even if a single locus may not follow the history of the species, due to introgression and incomplete lineage sorting (Puillandre et al. 2021), it nevertheless provides a first overview on the species-level diversity within a group.

Whether ASAP, GMYC or PTP, all these methods provide congruent results in suggesting about 23 highly divergent lineages, once the probably misleading lumping of MOTUs M13 and M14 in the mPTP analysis has been excluded (MOTUs M13 and M14 are separated by p-distances as high as 19.1% and appear to be joined by long branches in the ML tree, making their lumping into one hypothetical species questionable). In contrast, the bPTP approach provided some species hypotheses that are highly unlikely, e.g. splitting MOTUs with p-distances values as low as 2.1% (M1) and 3.5% (M12).

The performance of each method is variable and subject to its own errors, resulting in either oversplitting or overlumping (Dellicour and Flot 2018), and the GMYC approach is known to belong to the first category (Puillandre et al. 2021). In this respect, it is reassuring to see that ASAP, GMYC and, to a lesser extent PTP, all yielded

Table 2. Estimates of evolutionary divergence over sequence pairs between and within MOTUs (M) identified with the ASAP analysis (average uncorrected pairwise distances in per cent).

	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18	M19	M20	M21	M22	M23
M1	2.1																						
M2	20.0	2.0																					
M3	19.1	18.9	0.6																				
M4	18.5	21.2	18.8	n/c																			
M5	20.1	19.7	18.4	20.8	n/c																		
M6	21.5	21.3	22.3	23.3	20.4	0.3																	
M7	21.8	19.2	18.4	20.1	17.1	21.5	1.4																
M8	21.5	22.1	21.5	19.9	22.9	23.4	21.8	1.1															
M9	22.5	23.7	23.6	23.0	22.9	25.4	21.3	23.0	0.2														
M10	24.8	23.5	23.9	22.5	23.8	23.2	23.7	23.3	20.9	0.2													
M11	20.5	20.6	20.1	20.5	19.7	22.4	19.4	22.6	21.8	20.4	0.3												
M12	24.6	24.0	25.6	25.0	26.2	25.6	24.7	25.6	25.0	25.6	23.8	3.5											
M13	23.7	23.3	21.2	21.0	24.5	26.1	25.0	21.7	23.3	24.4	22.1	22.8	n/c										
M14	23.5	22.6	25.1	22.4	22.8	23.0	23.1	22.6	24.3	23.1	22.4	23.1	19.1	n/c									
M15	23.2	23.7	21.5	22.5	23.0	24.5	22.3	24.1	22.7	23.9	19.7	22.1	19.3	19.1	0.5								
M16	21.7	23.9	23.1	23.3	21.9	24.6	22.2	24.3	23.7	26.1	24.0	24.4	22.2	23.3	25.0	n/c							
M17	24.2	23.3	23.4	22.3	22.3	24.6	24.5	23.7	25.7	25.5	22.8	24.9	24.2	24.2	22.5	21.1	n/c						
M18	23.3	23.3	22.5	25.1	24.6	26.1	22.9	27.5	24.7	26.0	25.6	27.5	25.8	23.9	25.1	21.6	21.7	2.4					
M19	26.8	24.2	24.4	24.3	25.7	25.1	25.5	25.4	25.6	23.7	23.7	27.3	25.7	23.8	25.4	22.6	24.8	24.5	n/c				
M20	23.2	24.3	23.0	22.9	22.0	25.7	22.1	24.7	23.9	26.0	22.5	26.3	21.9	24.1	21.9	19.9	22.2	23.9	21.6	n/c			
M21	22.3	23.9	22.7	21.9	22.5	24.1	22.5	22.6	26.3	24.2	22.4	24.9	23.4	25.4	24.8	21.1	23.7	25.6	19.9	19.6	0.8		
M22	26.1	26.8	24.7	22.6	25.8	24.9	24.7	23.1	27.2	27.5	23.3	25.0	24.1	24.9	26.7	21.1	23.2	24.5	25.0	24.5	23.8	3.5	
M23	25.7	26.4	26.9	25.4	26.1	27.1	26.9	24.8	27.5	25.7	26.7	23.6	24.0	26.1	24.5	23.6	23.7	25.1	24.6	25.0	25.6	24.3	2.5

congruent results. This congruence can be interpreted as compelling evidence for the reliability of the outcomes obtained. With regard to the PTP approach, it is interesting to note that mPTP produces results closer to those of ASAP and GMYC than bPTP. This is consistent with the observation that mPTP is superior to PTP in producing delimitations more congruent with taxonomy (Kapli et al. 2017).

Although it is not advisable to consider MOTUs as distinct species on the basis of the mitochondrial COI alone, without at least including a nuclear marker (Dellicour and Flot 2018), several evidences suggest that most, if not all MOTUs identified by the congruence of the different methods used herein corresponds to a valid species.

The first piece of evidence is the particularly high mean p-distances between MOTUs (17.1 to 27.5%). Based on a dataset including a wide taxonomic coverage of Crustacea, Lefébure et al. (2006) have suggested that two monophyletic groups divergent by more than 0.16 substitution per site in the COI gene, as measured by patristic distances, have a strong probability to belong to different species. Patristic distances are defined as the amount of divergence since two taxa shared a common ancestor, i.e., the path-length distance between the two taxa along a phylogenetic tree. Later, Morvan et al. (2013) showed that the threshold method of Lefébure et al. (2006) applied to Aselloidea remained relevant. In this study, mean p-distances between *Metastenasellus* MOTUs are well above this threshold. This observation makes even more sense given that patristic distances are necessarily higher than p-distances because, unlike the latter, they take account of multiple substitutions. It should also be noted that the separation between the lineages must probably be ancient, as suggested by the particularly large distances between MOTUs, which would make the biases usually associated with the use of a single locus for species delimitation (such as introgression or incomplete sorting of lineages), all the more unlikely. It should be noted, however, that Raupach et al. (2022) have recently shown the existence of surprisingly high genetic divergences in the DNA barcode fragment (COI) within some woodlouse species, which it seems difficult to attribute to the existence of cryptic species, an observation that requires, however, future confirmation with nuclear markers.

Second evidence is the observation that two morphologically distinct species, *M. boutini* (M15) and *M. camerounensis* (M23), are separated by p-distances of 24.5%, i.e., a mean interspecific distance of the same order of magnitude as the distances between most other MOTUs (Table 2). An ongoing morphological study also shows that specimens from the same faunistic samples as MOTUs 9, 10 and 11 have clearly distinct male pleopods 2, confirming that these MOTUs correspond to distinct species (Lagnika, pers. comm.). These three MOTUs are separated by p-distances ranging from 20.4 to 24.8% (Table 2).

Third piece of evidence is the coexistence of two MOTUs in the same station, at the same time (BEN072: M6, M7; IBT: M1, M11) indicating that these MOTUs are separately evolving lineages, in other words potential distinct species according to the de Queiroz's species concept (de Queiroz 2007). However, it is important to keep in mind that divergent mitogenomes found in sympatry are not always associated with divergent nuclear sequences (Martinsson et al. 2020) and may coexist in a single species (Giska et al. 2015).

Diversity and endemism in *Metastenasellus*

A high level of species diversity in the genus *Metastenasellus* in Benin and Cameroon, with about 23 potential distinct species, is all the more remarkable given that, although this is the first significant sampling effort in these two countries, it is still very limited given the geographic area of these countries. However, it is not really a surprise given the recent realisation that the aquatic groundwater environment harbours rich macrobiological diversity, with a high level of endemism and numerous relict species (Gibert et al. 1994; Dole-Olivier et al. 2005; Gibert et al. 2009; Halse et al. 2014; Borko et al. 2021).

Although this study focused on a limited number of *Metastenasellus* stations, the distribution maps (Figs 2, 4) show a narrow geographical distribution of all MOTUs, suggesting high levels of endemism. The coexistence of two MOTUs in the same station remains the exception, but this may result from the limited sampling effort. In groundwater habitats, exceptional levels of endemism are generally assumed to be caused by strong hydrogeographical isolation, resulting in vicariance, and low dispersal abilities of their inhabitants (Gibert et al. 2009; Iepure et al. 2021). Following an exhaustive analysis of the literature on Stenasellidae in Africa, Pountougnigni et al. (2021) had already observed that the known distribution of this isopod family on this continent was very patchy, with most species known only from their type localities. It is generally accepted that hyporheic habitats of rivers can act as dispersal corridors for subterranean aquatic animals (Stanford and Ward 1993; Malard et al. 2023). In Benin, although the Ouémé river could have played this role, no MOTU from the upper part of the Ouémé basin was observed in the lower part of the basin, which suggests significant faunal isolation.

In Europe, Trontelj et al. (2009) suggested that macro-stygobiotic species showing range sizes over 200 km were most likely an assemblage of cryptic species with much smaller geographic ranges. Our data do not contradict this observation for this region of Africa as well. If so, we can expect future studies to reveal levels of diversity of stygobiotic isopods as high as those documented in other parts of the world. In Western Europe alone, Morvan et al. (2013) identified about 150 species and 12 genera of obligate groundwater Aselloidea (values corrected excluding groundwater species from USA, Japan, Lebanon and Mexico). This observation alone shows the extent to which the diversity of Aselloidea in Africa is probably unknown and remains to be discovered.

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What`s crawling in the dark? An annotated list of gastropods in Greek caves

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Abstract

Greece is covered by a high percentage of carbonate rocks, resulting in a significant number of over 10,000 caves within its territory. Their fauna is not well known. Concerning gastropods, 68 terrestrial species have been reported from 70 caves. In this study, we contribute to the knowledge of the snails inhabiting Greek caves. We analyzed the literature and studied all the material deposited in the Natural History Museum of Crete, to create a first comprehensive list of gastropods of Greek caves. The number of caves from which gastropod species were reported increased to 182, while the number of known terrestrial species increased to 113. Fourteen of these species live only in caves, twelve of which are endemic of Greece. However, the fauna of Greek caves is still understudied. With the increasing exploration of Greek caves, the number of the known cave-dwelling gastropod species will increase and new species will be discovered.

Keywords

Biodiversity, cave fauna, gastropoda, Greece, snails

Introduction

Karst terrains and karstic processes are significant components of the physical geography of the Mediterranean basin. A substantial percentage of the land area of Greece is covered by carbonate rocks, where most caves are formed. According to Chen et al. (2017), approximately 41% of the land surface of Greece is characterised by the

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presence of carbonate rocks. As a result, Greece, an area of 132,000 km^2, hosts more than 10,000 known caves (Beron 2016).

Among these caves, several hundred have been studied speleobiologically but faunistic data have been published only for 550. For terrestrial gastropods, 68 species have been reported from 70 caves (Paragamian et al. 2023). These species belong to 30 genera within 15 families, while half of them are Greek endemics (33/68). Fifty-nine of the species have been identified at species level; while 14 have been characterised as troglobionts, 30 as troglophiles and 15 as trogloxenes.

The classification of species living in subterranean environments has been a subject of debate [for a review, see Sket (2008, 2016) and Trajano and de Carvalho (2017)]. In this study, we refer to the terms of the Schiner-Racovitza's classification system, according to the lifestyle of the species as such: troglobiont, if a species lives exclusively in caves; troglophile, if it lives and reproduces in surface habitats but is also able to live and reproduce inside caves; trogloxene, if a species has an accidental presence in the cave or is considered an occasional visitor that does not live or reproduce in caves.

In this study, we contribute to the knowledge of the Greek cave gastropod fauna by presenting data from Greek caves based on the collections of the Natural History Museum of Crete (NHMC) and a thorough analysis of the literature.

Materials and methods

The present list is based on samples from caves in the collections of the NHMC and on the published data. The studied material is stored in the collections of the NHMC and consists of 96% ethanol-preserved specimens and dry material (empty shells). Most of the material was part of the collection of Kaloust Paragamian (KP, co-author) who donated his collection to the NHMC in 2018. It includes specimens from 137 caves and potholes (a single shaft, or an entire cave system that is dominantly vertical) that were collected between 1976 and 2019, mainly by KP but also by other Greek speleobiologists and cavers during their excursions (Fig. 1).

The majority of the specimens were identified at species level, following the taxonomy of MolluscaBase (2023). Where necessary, anatomical features of the reproductive system were also used for identification. In a few specimens, the anatomical characteristics were not clear due to preservation in 96% alcohol or the shells were broken. These specimens are reported at a genus level as *Genus* sp. or, if they resemble a specific species, they are reported as *Genus* aff. sp. (having affinity with but not identical to).

Additionally, we analysed the published data on cave-dwelling gastropods of Greece and included in the presented list all records reported as occurring in a cave. We excluded records, where it was uclear whether the species were collected inside or outside the cave (e.g. around the cave entrance).

For each species, we indicate the cave(s) in which it was found, based on the literature and the NHMC samples. The latter are reported as "present study". Note that if no other bibliographic references are given in a record, it is a new record for the Greek cave fauna.

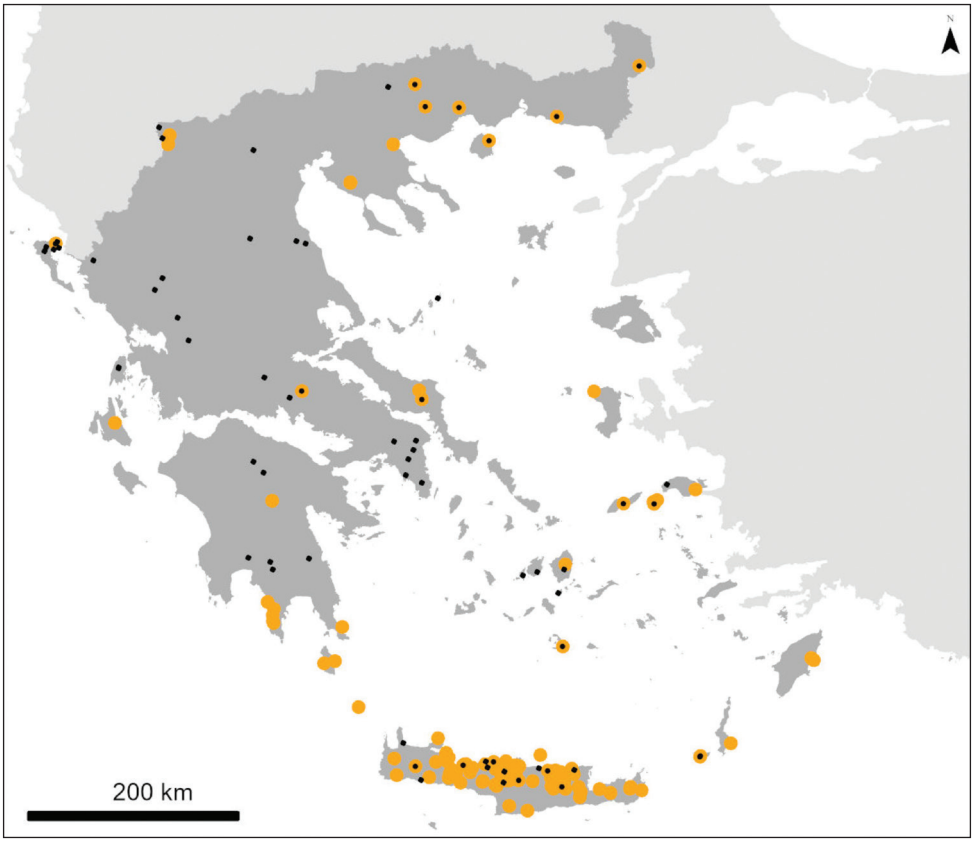

Figure 1. Caves in Greece with records of terrestrial gastropods from the literature (black dots) and from the present study (orange circles).

Sometimes the cave may have more than one name, in which case we follow the cave nomenclature of the Hellenic Institute of Speleological Research (Paragamian and Paragamian 2023), whereas the Greek letter transliteration is based on ELOT 743 standard.

The list is presented in alphabetical order of Order, Family, Genus and Species name. For each species, the cave(s) in which it is recorded, is given. The caves are presented in an alphabetical order of their TRADITIONAL GEOGRAPHIC REGION, Regional Unit and Cave name. However, for all islands, the island name is preferred to the Regional Unit, and they are divided into Aegean (including all islands of the Aegean Sea), Ionian (including all islands of the Ionian Sea) and Crete (including Crete island and its adjacent islets) areas (Fig. 2). The general distribution/endemism (Distribution), is also given for each species. Single-cave endemics, single-island endemics and stenoendemics (species with a few known localities within a very restricted area) are specifically mentioned. Finally, the ecological classification (Classification: Troglobiont, Troglophile, Trogloxene) and the published IUCN Red List category (IUCN 2023) are given for each species. The IUCN Red List categories are: Extinct (EX),

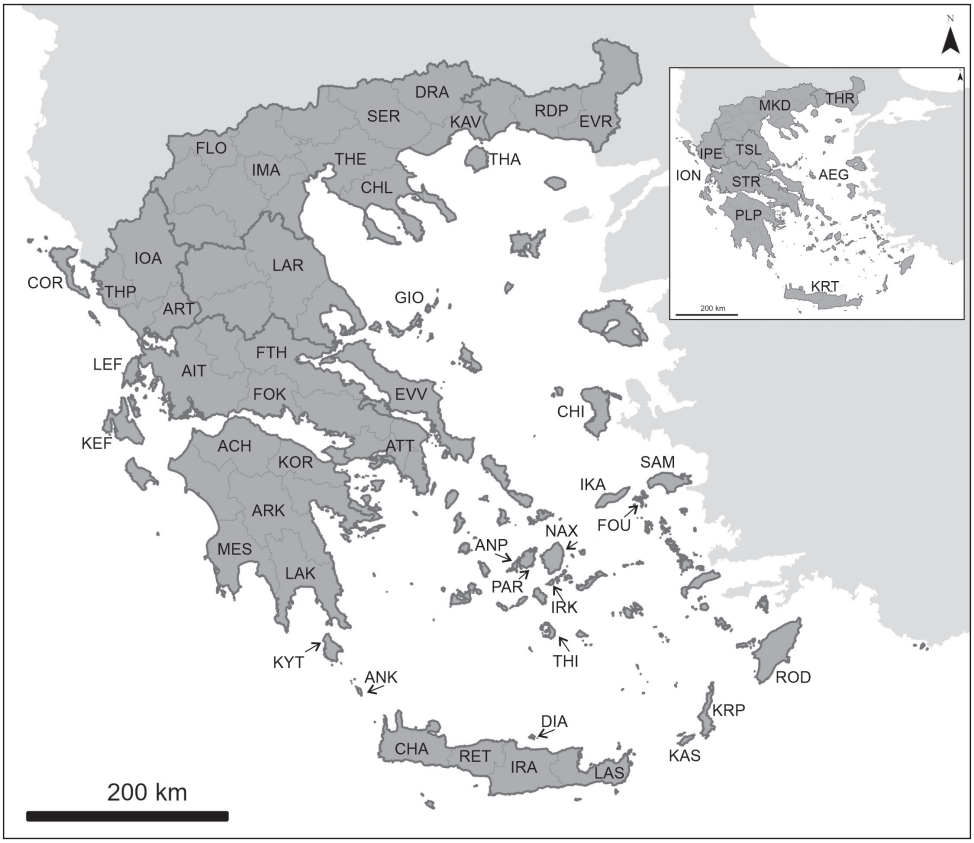

Figure 2. Map showing the Regional Units and Islands of Greece that are mentioned in the text. **Regional Units**: Achaia: **ACH**, Aitoloakarnania: **AIT**, Arkadia: **ARK**, Arta: **ART**, Attiki: **ATT**, Chalkidiki: **CHL**, Chania: **CHA**, Drama: **DRA**, Evros: **EVR**, Florina: **FLO**, Fokida: **FOK**, Fthiotida: **FTH**, Imathia: **IMA**, Ioannina: **IOA**, Irakleio: **IRA**, Kavala: **KAV**, Korinthia: **KOR**, Lakonia: **LAK**, Larisa: **LAR**, Lasithi: **LAS**, Messinia: **MES**, Rethymno: **RET**, Rodopi: **RDP**, Serres: **SER**, Thesprotia: **THP**, Thessaloniki: **THE**, Trikala: **TRI**. **Islands**: Antikythira: **ANK**, Antiparos: **ANP**, Chios: **CHI**, Corfu: **COR**, Dia: **DIA**, Evvoia: **EVV**, Fournoi: **FOU**, Gioura: **GIO**, Ikaria: **IKA**, Irakleia: **IRK**, Karpathos: **KRP**, Kasos: **KAS**, Kefalonia: **KEF**, Kythira: **KYT**, Lefkada: **LEF**, Naxos: **NAX**, Paros: **PAR**, Rodos: **ROD**, Samos: **SAM**, Thira: **THI**, Thasos: **THA**. Inset map showing the Traditional Geographic Regions of Greece. Aegean Islands: **AEG**, Crete: **KRT**, Ionian Islands: **ION**, Ipeiros: **IPE**, Makedonia: **MKD**, Peloponnisos: **PLP**, Sterea Ellada: **STR**, Thessalia: **TSL**, Thraki: **THR**.

Extinct In The Wild (EW), Critically Endangered (CR), Endangered (EN), Vulnerable (VU), Near Threatened (NT), Least Concern (LC) for evaluated species with adequate data, Data Deficient (DD) for evaluated species with inadequate data and Not Evaluated (NE) for not evaluated species. Detailed geographical information on the exact location of each cave is provided in Supplementary Material. The data underpinning the analysis reported in this paper are deposited at GBIF, the Global Biodiversity Information Facility, and are available at https://doi.org/10.15468/k57z2a.

Results

List of species in the caves of Greece

Terrestrial species

Phylum Mollusca
Class Gastropoda
Order Architaenioglossa
Family Cochlostomatidae
Genus *Cochlostoma*

***Cochlostoma cretense* (Maltzan, 1887)**

Distribution. Greek endemic, Crete (single-island endemic).
Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Crete: Irakleio: Lakki Kontari Pothole (present study).

Family Cyclophoridae
Genus *Pholeoteras*

***Pholeoteras euthrix* Sturany, 1904**

Distribution. Western Balkan.
Classification. Troglobiont.
IUCN RedList. LC.
Records in Greek caves. Ionian: Corfu: Grava Tsouka Cave (Gittenberger 1977, 1985, 1986; Bernasconi and Riedel 1994; Štamol et al. 1999; Beron 2016; Paragamian et al. 2023).

Order Stylommatophora
Family Argnidae
Genus *Speleodentorcula*

***Speleodentorcula beroni* Gittenberger, 1985**

Distribution. Greek endemic, Evvoia-Peloponnisos.
Classification. Troglobiont.
IUCN RedList. VU.
Records in Greek caves. Aegean: Evvoia: Skoteini Cave (Gittenberger 1985; Beron 2016; Paragamian et al. 2023; present study).

Notes. The species was until recently known only from its type locality. A new subspecies, *S. beroni maniates* Reischütz, Steiner-Reischütz & Reischütz, 2017, was described from a site 250 kilometres southwest from its type locality by Reischütz et al. (2017). Further research is needed to clarify the taxonomy and distribution of this genus.

Family Azecidae
Genus *Hypnophila*

***Hypnophila zacynthia* (Roth, 1855)**

Distribution. Greek endemic, Ionian Islands-Opposite Greek mainland.
Classification. Troglophile.
IUCN RedList. LC.
Records in Greek caves. Ionian: Corfu: Katsouri Cave (Sturany 1904; Gittenberger 1977, 2000; Beron 2016; Paragamian et al. 2023).

Family Chondrinidae
Genus *Granopupa*

***Granopupa granum* (Draparnaud, 1801)**

Distribution. Mediterranean-Macaronesian-Turanian.
Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Crete: Lasithi: Pelekita Cave (present study).

Genus *Rupestrella*

***Rupestrella rhodia* (Roth, 1839)**

Distribution. Eastern Mediterranean.
Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Aegean: Rodos: Koumelou Cave (present study).

Family Clausiliidae
Genus *Albinaria*

***Albinaria contaminata* (Rossmässler, 1835)**

Distribution. Greece-Albania.
Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Ionian: Kefalonia: Melissani Cave (present study).

***Albinaria corrugata* (Bruguière, 1792)**

Distribution. Greek endemic, Crete (single-island endemic).
Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Crete: Irakleio: Agia Paraskevi Cave (Frank 1988; Paragamian et al. 2023; present study), Neraidospilios Cave (Frank 1988; Paragamian et al. 2023).

***Albinaria discolor* (Pfeiffer, 1846)**

Distribution. Greek endemic, Aegean Islands-Sterea Ellada-Peloponnisos.
Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Aegean: Kythira: Katofygadi Cave (present study).

***Albinaria eburnea* (Pfeiffer, 1854)**

Distribution. Greek endemic, Crete (single-island endemic).
Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Crete: Chania: Drakolaki Cave (present study).

***Albinaria freytagi* (Böttger, 1889)**

Distribution. Greek endemic, Aegean Islands.
Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Aegean: Fournoi: Votsos Agiou Charalampous Pothole, Megalos Votsos Panagias Pothole, Mikros Votsos Panagias Pothole (present study).

***Albinaria grayana* (Pfeiffer, 1846)**

Distribution. Greek endemic, Aegean Islands-Peloponnisos.
Classification. Trogloxene.
IUCN RedList. NT.
Records in Greek caves. Aegean: Kythira: Niorou Cave (present study).

***Albinaria munda* (Rossmässler, 1836)**

Distribution. Aegean Islands-Opposite Turkish mainland.
Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Aegean: Samos: Vrysoulia Cave (present study).

***Albinaria senilis* (Rossmässler, 1836)**

Distribution. Greek endemic, Ionian Islands-Opposite Greek mainland.
Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Ionian: Kefalonia: Melissani Cave (present study).

***Albinaria teres* (Olivier, 1801)**

Distribution. Greek endemic, Crete and adjacent islets.
Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Crete: Lasithi: Pelekita Cave (present study).

***Albinaria* sp.**

Records in Greek caves. Crete: Irakleio: Latsida sto Makry Lakki Pothole (present study).
Notes. The specimen could not be identified at species level because only a broken shell was collected, which belongs to a species of the genus *Albinaria*.

Genus *Carinigera*

***Carinigera buresi* (Wagner, 1928)**

Distribution. Greece-Bulgaria.
Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Makedonia: Kavala: Agia Eleni Cave; **Serres:** Alistrati Cave (present study).

Genus *Montenegrina*

***Montenegrina dofleini* (Wagner, 1928)**

Distribution. Western Balkan.
Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Makedonia: Florina: Panagia Eleousa Cave (Fehér and Szekeres 2016; Paragamian et al. 2023).

***Montenegrina sattmanni* Nordsieck, 1988**

Distribution. Greece-Albania-North Macedonia.

Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Makedonia: Florina: Kokkali Cave (Fehér and Szekeres 2016; Paragamian et al. 2023).

Genus *Sciocochlea*

***Sciocochlea collasi* (Sturany, 1904)**

Distribution. Greek endemic, Ionian Islands (single-cave endemic).
Classification. Troglobiont.
IUCN RedList. NT.
Records in Greek caves. Ionian: Corfu: Katsouri Cave (Sturany 1904; Wolf 1938; Gittenberger 1977, 1985, 1986, 2000; Beron 2016; Reischütz et al. 2016; Paragamian et al. 2023).

***Sciocochlea nordsiecki* Subai, 1993**

Distribution. Greece-Albania.
Classification. Troglobiont.
IUCN RedList. NT.
Records in Greek caves. Ipeiros: Thesprotia: Agios Neilos Cave (Subai 1993; Reischütz et al. 2016; Paragamian et al. 2023).
Notes. All known *Sciocochlea* material has been collected from deposits or debris originating from deep limestone crevices that are known, or thought to be, connected to caves. So far, no live specimens have been found (Reischütz et al. 2016).

Genus *Stigmatica*

***Stigmatica stigmatica* (Rossmässler, 1836)**

Distribution. Balkan-Italy.
Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Ionian: Kefalonia: Melissani Cave (present study).

Genus *Tsoukatosia*

***Tsoukatosia christinae* Reischütz & Reischütz, 2003**

Distribution. Greek endemic, Peloponnisos (stenoendemic).
Classification. Troglobiont.
IUCN RedList. LC.

Records in Greek caves. Peloponnisos: Messinia: Agioi Anargyroi Cave (Reischütz and Reischütz 2003; Reischütz et al. 2016, 2020; Paragamian et al. 2023).

Notes. No specimen of the genus *Tsoukatosia* has been recorded alive. The type locality of *T. christinae* is the indicated Agioi Anargyroi Cave, but empty shells have been recorded in other sites within coarse gravel. It is not known whether the species lives subterranean or in caves (Reischütz et al. 2020).

Family Enidae
Genus *Napaeopsis*

***Napaeopsis ossica* (Böttger, 1885)**

Distribution. Greek endemic, Makedonia-Thessalia.

Classification. Trogloxene.

IUCN RedList. LC.

Records in Greek caves. Thessalia: Larisa: Kokkinos Vrachos Cave (Stussiner and Böttger 1885; Bank and Menkhorst 1992; Paragamian et al. 2023).

Notes. All literature references are based on specimens from the Senckenberg Museum Frankfurt (SMF), but according to Bank and Menkhorst (1992) the specimens of *N. ossica* from this cave do no longer exist. The presence of this species from Kokkinos Vrachos Cave needs confirmation.

Genus *Rhabdoena*

***Rhabdoena cosensis* (Reeve, 1849)**

Distribution. Aegean Islands-Opposite Turkish mainland.

Classification. Trogloxene.

IUCN RedList. LC.

Records in Greek caves. Aegean: Fournoi: Votsos sta Chalara Pothole (present study).

Genus *Mastus*

***Mastus sitiensis* Maassen, 1995**

Distribution. Greek endemic, Crete and adjacent islets.

Classification. Trogloxene.

IUCN RedList. LC.

Records in Greek caves. Crete: Lasithi: Chonos Pothole (present study).

Genus *Zebrina*

***Zebrina detrita* (Müller, 1774)**

Distribution. European.
Classification. Trogloxene.
IUCN RedList. NE.
Records in Greek caves. Makedonia: Florina: Daoula Pothole; **Serres:** Alistrati Cave (present study). **Thessalia: Larisa:** Kokkinos Vrachos Cave (Stussiner and Böttger 1885; Bank and Menkhorst 1992; Paragamian et al. 2023).

Family Ferussaciidae
Genus *Cecilioides*

***Cecilioides acicula* (Müller, 1774)**

Distribution. Euro-Mediterranean-Turanian-Macaronesian.
Classification. Troglophile.
IUCN RedList. LC.
Records in Greek caves. Aegean: Antikythira: Pano Spiliarida Cave; **Rodos:** Karolos Cave, Koumelou Cave (present study).

***Cecilioides michoniana* (Bourguignat, 1864)**

Distribution. Greece-Turkey.
Classification. Troglophile.
IUCN RedList. NE.
Records in Greek caves. Aegean: Rodos: Koumelou Cave (present study).

***Cecilioides tumulorum* (Bourguignat, 1856)**

Distribution. Mediterranean.
Classification. Troglophile.
IUCN RedList. LC.
Records in Greek caves. Aegean: Rodos: Koumelou Cave. **Crete: Lasithi:** Pelekita Cave, Theriospilios Cave. **Peloponnisos: Lakonia:** Agios Andreas Cave, Koukouri Cave; **Messinia:** Nerospiliako Cave (present study).

***Cecilioides* sp.**

Records in Greek caves. Ionian: Corfu: Kerkyra caves (Gittenberger 1977; Paragamian et al. 2023).

Family Gastrodontidae
Genus *Zonitoides*

***Zonitoides nitidus* (Müller, 1774)**

Distribution. Holarctic.
Classification. Troglophile.
IUCN RedList. NE.
Records in Greek caves. Makedonia: Imathia: Apano Skala Cave (Riedel 1959, 1992; Beron 2016; Paragamian et al. 2023).

Family Helicidae
Genus *Helix*

Helix* aff. *lucorum

Records in Greek caves. Thraki: Rodopi: Maroneia Cave (present study).

Genus *Cantareus*

***Cantareus apertus* (Born, 1778)**

Distribution. Mediterranean.
Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Crete: Irakleio: Arkalospilios Cave (present study).

Family Helicodontidae
Genus *Lindholmiola*

***Lindholmiola barbata* (Férussac, 1821)**

Distribution. Greek endemic, Crete and adjacent islets.
Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Crete: Chania: Achyrospilios Cave, Lentaka Trypa Cave (present study); **Rethymno**: Gerani Cave (Subai and Neubert 2014; Paragamian et al. 2023; present study).

***Lindholmiola corcyrensis* (Rossmässler, 1838)**

Distribution. Balkan.
Classification. Trogloxene.
IUCN RedList. LC.

Records in Greek caves. Ionian: Kefalonia: Melissani Cave (present study). **Ipeiros: Thesprotia:** Agios Neilos Cave (Subai and Neubert 2014; Paragamian et al. 2023).

***Lindholmiola lens* (Férussac, 1832)**

Distribution. Greece-W Turkey.
Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Ionian: Kefalonia: Melissani Cave (present study). **Sterea Ellada: Fthiotida:** Mavri Troupa Cave (Subai and Neubert 2014; Paragamian et al. 2023). **Peloponnisos: Achaia**: Limnon Cave (Subai and Neubert 2014; Paragamian et al. 2023); **Arkadia:** Small caves near Agios Dimitrios (Subai and Neubert 2014; Paragamian et al. 2023); **Lakonia:** Agios Andreas Cave (present study).

***Lindholmiola spectabilis* Urbański, 1960**

Distribution. Greek endemic, Makedonia.
Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Makedonia: Serres: Alistrati Cave (present study).

Family Hygromiidae
Genus *Metafruticicola*

***Metafruticicola nicosiana* (Mousson, 1854)**

Distribution. Eastern Mediterranean.
Classification. Trogloxene.
IUCN RedList. DD.
Records in Greek caves. Crete: Chania: Amoutses Cave (present study).

***Metafruticicola noverca* (Pfeiffer, 1853)**

Distribution. Greek endemic, Aegean Islands and Crete
Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Crete: Chania: Lentaka Trypa Cave, Metaxari Cave (present study).

***Metafruticicola redtenbacheri* (Pfeiffer, 1856)**

Distribution. Eastern Mediterranean.
Classification. Trogloxene.
IUCN RedList. LC.

Records in Greek caves. Aegean: Chios: Agio Gala Cave; **Fournoi:** Votsos sta Chalara Pothole (present study).

***Metafruticicola sublecta* (Maltzan, 1884)**

Distribution. Greek endemic, Crete and adjacent islets.
Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Crete: Irakleio: Latsida sto Vathy Lakko I Pothole (present study).

Genus *Monacha*

***Monacha parumcincta* (Menke, 1828)**

Distribution. Balkan-Italy.
Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Ionian: Kefalonia: Melissani Cave (present study).

Family Geomitridae
Genus *Xerocrassa*

***Xerocrassa* sp.**

Records in Greek caves. Aegean: Rodos: Koumelou Cave. **Crete: Lasithi:** Pelekita Cave (present study).

Genus *Xerolenta*

***Xerolenta obvia* (Menke, 1828)**

Distribution. European.
Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Makedonia: Serres: Alistrati Cave (present study).

Family Lauriidae
Genus *Lauria*

***Lauria cylindracea* (Da Costa, 1778)**

Distribution. Euro-Mediterranean-Turanian.

Classification. Trogloxene.
IUCN RedList. NE.
Records in Greek caves. Aegean: Fournoi: Megalos Votsos Panagias Pothole (present study).

Family Limacidae
Genus *Limacus*

***Limacus flavus* Linnaeus, 1758**

Distribution. Euro-Mediterranean.
Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Thraki: Rodopi: Maroneia Cave (present study).

Genus *Limax*

***Limax conemenosi* Böttger, 1882**

Distribution. Balkan.
Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Aegean: Samos: Panagia Spiliani Cave (present study).

***Limax graecus* Simroth, 1889**

Distribution. Balkan-Italy.
Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Makedonia: Chalkidiki: Nychteridon Cave; **Kavala:** Agia Eleni Cave; **Thraki: Rodopi:** Maroneia Cave (present study).
Notes. A slug was collected and identified as *Limax* aff. *graecus* in Petralona Cave (Makedonia: Chalkidiki) (present study).

***Limax* sp.**

Records in Greek caves. Crete: Irakleio: Afentis Christos Pothole (present study). **Ipeiros: Arta:** Trypa Rendes Cave (Beron 2016; Paragamian et al. 2023). **Sterea Ellada: Aitoloakarnania:** Gouva II Cave (Bonzano and Calandri 1984; Beron 2016; Paragamian et al. 2023)
Notes. Two species referred as *Limax maximus* var. *carbonaria* and *Limax maximus* var. *submaculata* are reported from **Thessalia: Larisa:** Kokkinos Vrachos Cave (Stussiner and Böttger 1885; Paragamian et al. 2023). Their taxonomy is not clear and their presence has never been confirmed since their description.

Family Milacidae
Genus *Tandonia*

***Tandonia cretica* (Simroth, 1884)**

Distribution. Greek endemic, Greece.
Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Crete: Irakleio: Chamoto Spiliari Cave, Aspalathospilios Cave; **Lasithi**: Tafos Cave (present study).

***Tandonia totevi* (Wiktor, 1975)**

Distribution. Greece-Bulgaria.
Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Makedonia: Kavala: Agia Eleni Cave. **Thraki: Rodopi:** Maroneia Cave (present study).

***Tandonia* sp.**

Records in Greek caves. Crete: Chania: Kourna Cave; **Irakleio:** Latsida sto Vathy Lakko I Pothole, Latsida ston Gkremismeno Cave, Latsida tou Siganou Cave, Trachinolakka Pothole; **Rethymno:** Diplotafki Pothole, Gerani Cave, Profitis Ilias Cave, Sipouli Pothole; **Dia islet:** Petrokotsyfou Pothole (present study).

Family Oleacinidae
Genus *Poiretia*

***Poiretia compressa* (Mousson, 1859)**

Distribution. Greece-Albania.
Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Ionian: Corfu: Anonymous cave at Pantokrator Mt. (Käufel 1930; Paragamian et al. 2023).

Family Orculidae
Genus *Orcula*

? *Orcula* sp.

Records in Greek caves. Aegean: Thira: Zoodochou Pigis Cave (Beron 2016; Paragamian et al. 2023).

Notes. This record needs confirmation, since the genus is absent from the Aegean islands. Most likely it is a misidentification.

Genus *Orculella*

***Orculella exaggerata* (Fuchs & Käufel, 1936)**

Distribution. Greek endemic, Aegean Islands.
Classification. Troglophile.
IUCN RedList. LC.
Records in Greek caves. Aegean: Kasos: Stylokamara Cave (Gittenberger and Hausdorf 2004; Paragamian et al. 2023).

***Orculella ignorata* Hausdorf, 1996**

Distribution. Greece-Turkey.
Classification. Troglophile.
IUCN RedList. LC.
Records in Greek caves. Aegean: Fournoi: Votsos Agiou Theologou Pothole (present study).

***Orculella* sp.**

Records in Greek caves. Crete: Lasithi: Pelekita Cave (present study).
Notes. Only juvenilles collected. Around the cave, *Orculella cretiminuta* Gittenberger & Hausdorf, 2004 is present.

Family Oxychilidae
Genus *Carpathica*

***Carpathica cretica* (Forcart, 1950)**

Distribution. Eastern Mediterranean.
Classification. Trogloxene.
IUCN RedList. LC.
Records in Greek caves. Crete: Irakleio: Agia Paraskevi Cave (present study); **Rethymno**: Gerani Cave (Riedel 1977, 1992; Beron 2016; Paragamian et al. 2023).

***Carpathica insularis* Riedel & Mylonas, 1988**

Distribution. Greek endemic, Aegean Islands.
Classification. Troglophile.
IUCN RedList. LC.

Records in Greek caves. Aegean: Fournoi: Megalos Votsos Panagias Pothole (Riedel 1988a, Riedel 1992; Paragamian et al. 2023).

Genus *Daudebardia*

***Daudebardia brevipes* (Draparnaud, 1805)**

Distribution. European.
Classification. Trogloxene.
IUCN RedList. NE.
Records in Greek caves. Crete: Rethymno: Gerani Cave (Riedel 1977, 1992; Beron 2016; Paragamian et al. 2023).

***Daudebardia rufa* (Draparnaud, 1805)**

Distribution. European.
Classification. Trogloxene.
IUCN RedList. NE.
Records in Greek caves. Aegean: Evvoia: Katavothra Pothole (present study). **Sterea Ellada: Attiki:** Panos Cave (Riedel 1978; Riedel 1992; Paragamian et al. 2023). **Crete: Chania:** Tzani Spilios Cave; **Irakleio:** Lakki Kontari Pothole (present study); **Rethymno:** Gerani Cave (Riedel 1977, 1992; Beron 2016; Paragamian et al. 2023). **Peloponnisos: Arkadia:** Kapsia Cave (present study).

Genus *Eopolita*

***Eopolita protensa* (Férussac, 1832)**

Distribution. Eastern Mediterranean.
Classification. Troglophile.
IUCN RedList. NE.
Records in Greek caves. Aegean: Rodos: Koufovouni Pothole, Koumelou Cave (present study). **Sterea Ellada: Attiki:** Nymfolyptou Cave (Riedel 1959; Beron 2016; Paragamian et al. 2023), Rachi I Cave (Riedel 1959; Paragamian et al. 2023). **Crete: Chania:** Agia Sofia Cave (present study), Kolympari Cave (Riedel 1968; Beron 2016; Paragamian et al. 2023), Kourna Cave; **Irakleio:** Afentis Christos Pothole, Anonymous cave at Chersonisos, Anonymous Latsida of Geraki Pothole, Chainospilios Cave, Chamoto Spiliari Cave, Fragkadoni Cave, Karvounolakos Pothole, Lakki Kontari Pothole, Meires Trypa Cave, Palmeti Tafkos Pothole; **Lasithi:** Achnistra Cave (present study); **Rethymno:** Idaio Antro Cave (Riedel 1968; Paragamian et al. 2023), Mythia Kampathoura Pothole (present study).

Genus *Mediterranea*

***Mediterranea amaltheae* (Riedel & Subai, 1982)**

Distribution. Greek endemic, Crete (single-island endemic).

Classification. Troglobiont.

IUCN RedList. CR.

Records in Greek caves. Crete: Irakleio: Faneromeni Cave, Latsida ston Gkremismeno Cave (present study); **Lasithi:** Diktaio Antro Cave (Riedel and Subai 1982; Riedel 1992, 1996; Beron 2016; Paragamian et al. 2023, present study), Gaidourotrypa Cave, Peristeras Pothole, Tou Sfakianou i Trypa Pothole, Latsida Bempona Pothole (present study).

Notes. A remarkable extension of the distribution of *M. amaltheae* is reported. Until now, the species was known only from its type locality, Diktaio Antro Cave in Dikti Mt. (Lasithi, Crete). It is found in six other caves on the island and is therefore no longer a single-cave endemic but an island endemic.

***Mediterranea depressa* (Sterki, 1880)**

Distribution. Euro-Mediterranean.

Classification. Troglophile.

IUCN RedList. LC.

Records in Greek caves. Makedonia: Kavala: Agia Eleni Cave (Riedel 1992; Beron 2016; Paragamian et al. 2023), Mavri Trypa Cave (Riedel 1992; Beron 2016; Paragamian et al. 2023).

***Mediterranea hydatina* (Rossmässler, 1838)**

Distribution. European.

Classification. Troglophile.

IUCN RedList. LC.

Records in Greek caves. Aegean: Antiparos: Antiparos Cave (Riedel 1983, 1992, 1996; Beron 2016; Paragamian et al. 2023); **Fournoi:** Votsos Agiou Charalampous Pothole (present study), Megalos Votsos Panagias Pothole (Riedel 1992, 1996; Paragamian et al. 2023; present study); **Ikaria:** Foutra Raos Cave (Martens 1889; Riedel 1983, 1992, 1996; Paragamian et al. 2023; present study); **Kasos:** Ellinokamara Cave (Riedel 1992; Beron 2016; Paragamian et al. 2023); **Paros:** Kalampaki Cave (Riedel 1992; Beron 2016; Paragamian et al. 2023); **Rodos:** Koufovouni Pothole (present study). **Crete: Chania:** Kolympari Cave (Riedel 1968, 1992; Beron 2016; Paragamian et al. 2023), Tzani Spilios Cave (Riedel 1992, 1996; Beron 2016; Paragamian et al. 2023); **Irakleio:** Afentis Christos Pothole, Latsida ston Gkremismeno Cave. **Makedonia: Florina:** Mikrolimni Cave (present study); **Serres:** Alistrati Cave (Riedel 1992, 1996; Beron 2016; Paragamian et al. 2023; present study); **Thessaloniki:** Drakotrypa Cave (present study).

***Mediterranea ionica* (Riedel & Subai, 1978)**

Distribution. Balkan-Italy.

Classification. Troglophile.

IUCN RedList. LC.

Records in Greek caves. Ionian: Corfu: Anonymous cave at Pantokrator Mt. (Käufel 1930; Riedel 1992, 1996; Paragamian et al. 2023), Katsouri Cave (Riedel 1978, 1992, 1996; Riedel and Subai 1982; Paragamian et al. 2023). **Ipeiros: Ioannina:** Anemotrypa Cave (Riedel 1992, 1996; Paragamian et al. 2023); **Thesprotia:** Agios Neilos Cave (Riedel and Subai 1993; Paragamian et al. 2023).

***Mediterranea juliae* (Riedel, 1990)**

Distribution. Greece-Albania.

Classification. Troglophile.

IUCN RedList. LC.

Records in Greek caves. Ionian: Corfu: Megali Grava Cave (Riedel 1990, 1992, 1996; Beron 2016; Paragamian et al. 2023; present study).

Notes. Riedel and Subai (1993) reported this species from Agios Neilos Cave (Ipeiros, Thesprotia) but they note that its presence needs to be confirmed by anatomical data.

***Mediterranea* ? *mylonasi* (Riedel, 1983)**

Distribution. Greek endemic, Aegean Islands.

Classification. Troglophile.

IUCN RedList. LC.

Records in Greek caves. Aegean: Evvoia: Skoteini Cave (Riedel 1993, 1996; Beron 2016; Paragamian et al. 2023); **Gioura:** Kyklopa Cave (Riedel 1992, 1996; Beron 2016; Paragamian et al. 2023); **Samos:** Panagia Spiliani Cave (present study).

Notes. The taxonomy of the species is uncertain (Riedel 1992).

***Mediterranea* sp.**

Records in Greek caves. Ipeiros: Ioannina: Platanousa Cave, (Klemm 1962 as *Oxychilus eudedaleus*; Riedel 1979, 1992 as *Oxychilus* (*Riedelius* ?) sp. (nova?); Beron 2016 as *Oxychilus* (*Riedelius*?) sp. (nova?); Paragamian et al. 2023 as *Oxychilus* sp.).

Genus *Morlina*

***Morlina glabra* (Rossmässler, 1835)**

Distribution. European.

Classification. Troglophile.

IUCN RedList. LC.

Records in Greek caves. Aegean: Evvoia: Katavothra Pothole (present study); **Thasos:** Drakotrypa Cave (Riedel 1992; Beron 2016; Paragamian et al. 2023; present study). **Makedonia: Drama:** Maara Cave; **Florina:** Mikrolimni Cave (present study); **Imathia:** Apano Skala Cave (Riedel 1959; Beron 2016; Paragamian et al. 2023); **Kavala:** Agia Eleni Cave (present study), Mavri Trypa Cave (Riedel 1992; Beron 2016; Paragamian et al. 2023; present study); **Serres:** Pestereta Cave (Riedel 1959; Beron 2016; Paragamian et al. 2023); **Thessaloniki:** Drakotrypa Cave (present study). **Thessalia: Larisa:** Kokkinos Vrachos Cave (Stussiner and Böttger 1885; Riedel 1992; Beron 2016; Paragamian et al. 2023).

Genus *Oxychilus*

***Oxychilus cyprius* (Pfeiffer, 1847)**

Distribution. Eastern Mediterranean.

Classification. Troglophile.

IUCN RedList. NE.

Records in Greek caves. Makedonia: Imathia: Apano Skala Cave (Riedel 1959, 1983, 1992; Beron 2016; Paragamian et al. 2023).

***Oxychilus seidli* Riedel, 1999**

Distribution. Aegean Islands-Opposite Turkish mainland.

Classification. Troglophile.

IUCN RedList. LC.

Records in Greek caves. Aegean: Fournoi: Votsos Agiou Charalampous Pothole, Megalos Votsos Panagias Pothole (present study).

***Oxychilus superfluus* (Pfeiffer, 1849)**

Distribution. Greek endemic, Crete and adjacent islets.

Classification. Troglophile.

IUCN RedList. LC.

Records in Greek caves. Crete: Irakleio: Chainospilios Cave, Latsida sto Vathy Lakko I Pothole (present study); **Rethymno**: Idaio Antro Cave (Riedel 1968; Paragamian et al. 2023).

***Oxychilus* sp.**

Records in Greek caves. Sterea Ellada: Fokida Zoodochos Pigi Cave (Alexiou et al. 2014), Mayer Cave (Alexiou et al. 2014; Paragamian et al. 2023). **Crete: Chania**: Agia Sofia Cave (present study), Kolympari Cave as **?** *Oxychilus subeffusus* (Riedel

1968, 1992; Beron 2016; Paragamian et al. 2023); **Irakleio:** Stou Bokou ton Poro Pothole; **Rethymno**: Katerianos Tafkos Pothole (present study). **Peloponnisos**: **Messinia:** Agioi Anargyroi Cave (Reischütz and Reischütz 2003; Paragamian et al. 2023).

Genus *Schistophallus*

***Schistophallus minoicus* (Riedel, 1968)**

Distribution. Greek endemic, Crete (single-island endemic).

Classification. Troglophile.

IUCN RedList. LC.

Records in Greek caves. Crete: Irakleio: Faneromeni Cave, Latsida sto Vathy Lakko I Pothole (present study); **Lasithi:** Diktaio Antro Cave (Riedel 1968, 1996; Maassen and Riedel 1991; Beron 2016; Paragamian et al. 2023, present study), Tou Sfakianou i Trypa Pothole (present study).

***Schistophallus samius* (Martens, 1889)**

Distribution. Greece-Turkey.

Classification. Troglophile.

IUCN RedList. NE.

Records in Greek caves. Aegean: Chios: Agio Gala Cave (present study), Cave in Chios (Martens 1889); **Fournoi:** Megalos Votsos Panagias Pothole (Riedel 1993, 1996; Paragamian et al. 2023); **Naxos:** Za Cave (Beron 2016; Paragamian et al. 2023); **Samos:** Cave on Kerketeas Mt. (Martens 1889; Riedel 1958, 1972, 1992, 1996; Beron 2016; Paragamian et al. 2023), Panagia Spiliani Cave (present study). **Sterea Ellada: Attiki**: Vredou Cave (Riedel 1972; Beron 2016; Paragamian et al. 2023). **Peloponnisos: Achaia:** Limnon Cave (Riedel 1986, 1992, 1996; Beron 2016; Paragamian et al. 2023); **Lakonia:** Katafygi Cave (Anavryti) (Riedel 1992, 1996; Paragamian et al. 2023); **Korinthia:** Cave at Saitas Mt. (Arndt and Subai 2012).

***Schistophallus spratti* (Westerlund, 1892)**

Distribution. Greek endemic, Crete (single-island endemic).

Classification. Trogloxene.

IUCN RedList. LC.

Records in Greek caves. Crete: Chania: Amoutses Cave, Arapi Trypa Pothole, Kourna Cave, Lentaka Trypa Cave, Metaxari Cave (present study), Tzani Spilios Cave (Riedel 1992, 1996; Beron 2016; Paragamian et al. 2023; present study), Zoures Cave (present study); **Irakleio:** Kynigotafkos Pothole, Poros Stenou Lagkou Pothole, Stou Bokou ton Poro Pothole (present study); **Rethymno:** Afroimou Cave, Dolonas Cave (present study), Gerani Cave (Riedel 1977, 1992, 1996; Beron 2016; Paragamian et al. 2023), Kaoudi Tafkos Pothole, Maryle Trypa Cave, Platani Cave, Tafkos Myristis Pothole, Tigania Cave, Voreini Trypa Cave, Skylogkremi Cave (present study).

Family Pagodulinidae
Genus *Pagodulina*

***Pagodulina* sp.**

Records in Greek caves. Peloponnisos: Messinia: Agioi Anargyroi Cave (Reischütz and Reischütz 2003; Paragamian et al. 2023).

Family Pleurodiscidae
Genus *Pleurodiscus*

***Pleurodiscus balmei* (Potiez & Michaud, 1838)**

Distribution. Eastern Mediterranean.
Classification. Troglophile.
IUCN RedList. LC.
Records in Greek caves. Aegean: Fournoi: Megalos Votsos Panagias Pothole, Mikros Votsos Panagias Pothole; **Samos:** Panagia Spiliani Cave, Vrysoulia Cave (present study).

***Pleurodiscus sudensis* (Pfeiffer, 1846)**

Distribution. Greek endemic, Crete and adjacent islets.
Classification. Troglophile.
IUCN RedList. LC.
Records in Greek caves. Crete: Irakleio: Chainospilios Cave, Chonos Sarchou Cave, Anonymous Latsida of Geraki Pothole, Chamoto Spiliari Cave, Fragkadoni Cave, Doxa Cave, Arkalospilios Cave, Faneromeni Cave, Karvounolakkos Cave, Karvounolakos Pothole, Lakki Kontari Pothole, Latsida of Moni Kaleri Pothole, Latsida sto Makry Lakki Pothole, Megalos Tafkos Pothole, Moni Kaleri Cave, Palmeti Tafkos Pothole, Peristerotafkos Pothole, Skararolithos Pothole, Stravomyti Cave, Trachinolakka Pothole, Xerakolagoufo Pothole; **Lasithi:** Trapeza Cave; **Rethymno:** Alykes Cave, Gerani Cave, Kamariti Cave, Karafti Tafkos Pothole, Katerianos Tafkos Pothole, Melidoni Cave, Peiraton Cave, Porou Trypa Cave, Skoteinospilios Cave, Stin Koryfi tis Viglas Pothole, Tafkos sti Gonia Pothole, Tafkos stin Pode Faragka Pothole, Trypiti Cave, Xepatomenos Tafkos Pothole (present study).

Family Pomatiidae
Genus *Pomatias*

***Pomatias elegans* (Müller, 1774)**

Distribution. Euro-Mediterranean.
Classification. Trogloxene.

IUCN RedList. LC.

Records in Greek caves. Ionian: Kefalonia: Melissani Cave (present study).

Family Pristilomatidae
Genus *Gyralina*

***Gyralina formosa* Riedel & Subai, 1993**

Distribution. Greek endemic, Ipeiros (single-cave endemic).

Classification. Troglobiont.

IUCN RedList. VU.

Records in Greek caves. Ipeiros: Thesprotia: Agios Neilos Cave (Riedel and Subai 1993).

Notes. The shells were found in rock debris, on limestone rocks in the small cave chapel (Riedel and Subai 1993). In the type locality the species is known from 10 empty shells, including 7 adults, some very fresh. The species may also occur in other subterranean environments other than caves (unpublished data). If this is confirmed, then the species should be considered as a subterranean, troglophilous species.

***Gyralina tsatsae* Gittenberger, 1977**

Distribution. Greek endemic, Ionian Islands (single-island endemic).

Classification. Troglobiont.

IUCN RedList. VU.

Records in Greek caves. Ionian: Corfu: Anthropograva Cave (Gittenberger 1977; Riedel 1992; Beron 2016; Paragamian et al. 2023), Grava Tsouka Cave (Gittenberger 1977; Riedel 1992; Beron 2016; Paragamian et al. 2023).

Genus *Lindbergia*

***Lindbergia beroni* Riedel, 1984**

Distribution. Greek endemic, Aegean Islands (single-cave endemic).

Classification. Troglobiont.

IUCN RedList. CR.

Records in Greek caves. Aegean: Thira: Zoodochou Pigis Cave (Riedel 1984, 1992, 1996; Beron 1985, 2016; Di Russo and Rampini 2001; Gasparo 2009; Paragamian et al. 2023; present study).

Notes. In the collections of the NHMC there are specimens of *Lindbergia* aff. *beroni* from Kanavari Cave (Aegean: Naxos).

***Lindbergia* ? *gittenbergeri* Pintér & Riedel, 1983**

Distribution. Greek endemic, Ionian Islands (single-island endemic).

Classification. Troglobiont.

IUCN RedList. CR.

Records in Greek caves. Ionian: Corfu: Megali Grava Cave (Pintér and Riedel 1983; Riedel 1990, 1992, 1996; Beron 2016; Paragamian et al. 2023), Anonymous cave (Riedel 1992; Beron 2016; Paragamian et al. 2023).

Notes. The presence of the genus on the island of Corfu requires confirmation (Riedel 1992).

Lindbergia aff. *karainensis*

Records in Greek caves. Aegean: Karpathos: Diaolospilios Cave (present study).

Lindbergia orbicularis (Riedel, 1962)

Distribution. Greek endemic, Crete (single-island endemic).

Classification. Troglophile.

IUCN RedList. LC.

Records in Greek caves. Crete: Chania: Agia Roumeli Cave (Riedel 1992, 1996; Paragamian et al. 2023), Metaxari Cave (present study), Tzani Spilios Cave (Riedel 1993, 1996; Beron 2016; Paragamian et al. 2023; present study); **Irakleio:** Agio Pnevma Cave, Anonymous Latsida of Geraki Pothole (present study), Chainospilios Cave (Riedel 1968, 1992, 1996; Beron 2016; Paragamian et al. 2023; present study), Doxa Cave, Karvounolakkos Cave, Lakki Kontari Pothole, Peristerotafkos Pothole, Schistra Cave, Skararolithos Pothole, Stou Bokou ton Poro Pothole, Trachinolakka Pothole, Xerakolagoufo Pothole (present study); **Lasithi:** Achnistres Cave, Peristera Cave (Agios Georgios), Peristera Cave (Vrachasi), Tafos Cave, Xepatomeni Latsida Pothole (present study); **Rethymno:** Chomatotafkos Pothole, Diplotafki Pothole, Erfoi Cave (present study), Gerani Cave (Riedel 1977, 1992, 1996; Beron 2016; Paragamian et al. 2023), Kakalonia Pothole, Kamariti Cave, Karafti Tafkos Pothole, Katerianos Tafkos Pothole (present study), Melidoni Cave (Riedel 1968, 1992, 1996; Beron 2016; Paragamian et al. 2023), Porou Trypa Cave, Tafkos stin Pode Faragka Pothole, Xanthou Cave, Xylouri Tafkos Pothole (present study).

Lindbergia ? *pageti* Riedel, 1968

Distribution. Greek endemic, Crete and adjacent islets.

Classification. Troglophile.

IUCN RedList. VU.

Records in Greek caves. Crete: Chania: Kolympari Cave (Riedel 1968, 1977, 1992, 1996; Beron 2016; Paragamian et al. 2023).

Notes. Previously, the species was reported with certainty only from the type locality—Kolympari Cave—and was considered to be a troglobiont. Specimens from other locations in Crete and its adjacent islands have been reported in the past but their identification was uncertain and therefore not confirmed by Riedel (1992). The collection

of the NHMC has a lot more material than the material Riedel had the opportunity to study and as a result, Mylonas was able to confirm the presence of the species in other localities on Crete island and Western Paximadi islet. A specimen from Tafos Cave (Crete: Lasithi), most probably belongs to this species, *Lindbergia* aff. *pageti.* Research on the taxonomy of the species is still needed, as there are no anatomical data to confirm the genus.

***Lindbergia pinteri* Riedel, 1981**

Distribution. Greek endemic, Aegean Islands (single-cave endemic).

Classification. Troglobiont.

IUCN RedList. VU.

Records in Greek caves. Aegean: Ikaria: Foutra Raos Cave (Riedel 1981a, 1992, 1996; Beron 2016; Paragamian et al. 2023).

***Lindbergia pseudoillyrica* Riedel, 1960**

Distribution. Greek endemic, Crete and adjacent islets.

Classification. Troglophile.

IUCN RedList. LC.

Records in Greek caves. Crete: Chania: Tzani Spilios Cave (Riedel 1992, 1996; Beron 2016; Paragamian et al. 2023); **Irakleio:** Achnistra Cave, Afentis Christos Pothole (present study), Agia Paraskevi Cave (Riedel 1960, 1968,1992, 1996; Beron 2016; Paragamian et al. 2023), Chonos Sarchou Cave (Riedel 1960, 1968, 1977, 1992, 1996; Beron 2016; Paragamian et al. 2023; present study), Meires Trypa Cave (present study); **Lasithi:** Agios Stavros Cave (present study), Diktaio Antro Cave (Riedel 1992, 1996; Beron 2016; Paragamian et al. 2023), Theriospilios Cave (present study); **Rethymno:** Peristere Cave (ancient quarry)(Riedel 1968, 1992, 1996; Beron 2016; Paragamian et al. 2023), Prinos Cave (Beron 2016; Paragamian et al. 2023), Sfentoni Trypa Cave (Riedel 1977, 1992, 1996; Beron 2016; Paragamian et al. 2023).

***Lindbergia spiliaenymphis* Riedel, 1959**

Distribution. Greek endemic, Sterea Ellada (single-cave endemic).

C**lassification.** Troglobiont.

IUCN RedList. VU.

Records in Greek caves. Sterea Ellada: Attiki: Keratea Cave (Riedel 1959, 1960, 1968, 1977, 1992, 1996; Pintér and Riedel 1983; Beron 2016; Paragamian et al. 2023).

***Lindbergia stylokamarae* Riedel, 1981**

Distribution. Greek endemic, Aegean Islands (single-island endemic).

Classification. Troglophile.

IUCN RedList. VU.

Records in Greek caves. Aegean: Kasos: Stylokamara Cave (Riedel 1981a, 1992; Beron 2016; Paragamian et al. 2023; present study).

***Lindbergia* sp.**

Records in Greek caves. Aegean: Irakleia: Agios Ioannis Cave, as *Lindbergia* sp. (nova) aff. *pseudoillyrica* (Riedel 1990, 1992, 1996; Beron 2016; Paragamian et al. 2023). **Peloponnisos: Messinia:** Agioi Anargyroi Cave (Reischütz and Reischütz 2003; Paragamian et al. 2023).

Genus *Vitrea*

***Vitrea clessini* (Hesse, 1882)**

Distribution. Greek endemic, Aegean Islands.

Classification. Troglophile.

IUCN RedList. LC.

Records in Greek caves. Aegean: Kasos: Ellinokamara Cave (present study).

***Vitrea contracta* (Westerlund, 1871)**

Distribution. Euro-Mediterranean-Turanian-Macaronesian.

Classification. Troglophile.

IUCN RedList. LC.

Records in Greek caves. Aegean: Fournoi: Megalos Votsos Panagias Pothole (Riedel 1992; Paragamian et al. 2023), Votsos Agiou Charalampous Pothole (Riedel 1992). **Crete: Lasithi:** Diktaio Antro Cave (Riedel 1992; Paragamian et al. 2023); **Rethymno:** Erfoi Cave (present study), Gerani Cave (Riedel 1977, 1992; Beron 2016; Paragamian et al. 2023). **Peloponnisos: Lakonia:** Agios Andreas Cave (present study), Kaiadas Cave (Riedel 1992; Paragamian et al. 2023).

***Vitrea klemmi* Pintér, 1972**

Distribution. Greek endemic, Aegean Islands (single-island endemic).

Classification. Troglophile.

IUCN RedList. LC.

Records in Greek caves. Aegean: Ikaria: Foutra Raos Cave (Riedel 1981a, 1981b, 1992; Beron 2016; Paragamian et al. 2023).

***Vitrea neglecta* Damjanov & Pintér, 1969**

Distribution. Greece-Bulgaria-Turkey.

Classification. Troglophile.

IUCN RedList. LC.

Records in Greek caves. Makedonia: Drama: Maara Cave (Georgiev 2016; Paragamian et al. 2023).

***Vitrea ossaea* Pintér, 1983**

Distribution. Greek endemic, Thessalia (single-cave endemic).

Classification. Troglobiont.

IUCN RedList. DD.

Records in Greek caves. Thessalia: Larisa: Profitis Ilias Cave (Pintér 1983; Riedel 1992; Riedel 1996; Beron 2016; Paragamian et al. 2023).

Notes. This species has also been reported from Peristera islet (Aegean) but the taxonomy of the specimen requires confirmation (Riedel 1992).

***Vitrea riedeli* Damjanov & Pintér, 1969**

Distribution. Greece-Bulgaria-Turkey.

Classification. Troglophile.

IUCN RedList. LC.

Records in Greek caves. Aegean: Fournoi: Megalos Votsos Panagias Pothole (Riedel 1992; Paragamian et al. 2023; present study).

***Vitrea riedeliana* Paget, 1976**

Distribution. Greece-Turkey.

Classification. Troglophile.

IUCN RedList. LC.

Records in Greek caves. Aegean: Rodos: Koufovouni Pothole, Koumelou Cave (present study).

***Vitrea schuetti* Pintér, 1972**

Distribution. Greek endemic, Greece.

Classification. Troglophile.

IUCN RedList. LC.

Records in Greek caves. Sterea Ellada: Attiki: Ntavelis Cave (Pintér 1972; Riedel 1992; Beron 2016; Paragamian et al. 2023).

***Vitrea subrimata* (Reinhardt, 1871)**

Distribution. Euro-Mahrebian.

Classification. Troglophile.

IUCN RedList. LC.

Records in Greek caves. Ipeiros: Thesprotia: Agios Neilos Cave (Riedel and Subai 1993).

Family Zonitidae
Genus *Allaegopis*

***Allaegopis jonicus* (Käufel, 1930)**

Distribution. Greek endemic, Ionian Islands-Opposite Greek mainland.

Classification. Trogloxene.

IUCN RedList. LC.

Records in Greek caves. Ionian: Lefkada: Egklouvi Cave (Käufel 1930; Riedel 1982, 1992; Beron 2016; Paragamian et al. 2023), Poly Trypes Cave (Käufel 1930; Riedel 1992; Paragamian et al. 2023).

Genus *Balcanodiscus*

***Balcanodiscus beroni* Riedel, 1995**

Distribution. Greek endemic, Makedonia (single-cave endemic).

Classification. Troglobiont.

IUCN RedList. NT.

Records in Greek caves. Makedonia: Drama: Maara Cave (Riedel 1995, 1996; Beron 2016; Paragamian et al. 2023).

Notes. In the studies of Riedel (1995) and Beron (2016), the species is reported from two caves, Maara I and Maara II. These are not two different caves, but part of the same cave system called Maara.

***Balcanodiscus cerberus* Riedel, 1985**

Distribution. Greek endemic, Thraki (stenoendemic).

Classification. Troglophile.

IUCN RedList. NT.

Records in Greek caves. Thraki: Rodopi: Maroneia Cave (Riedel 1985b, 1988b, 1992, 1996; Reischütz 1986, 1988; Paragamian et al. 2004, 2023; Beron et al. 2004; Erőss et al. 2011; Beron 2016; present study).

***Balcanodiscus difficilis* Riedel, 1988**

Distribution. Greek endemic, Aegean Islands (single-island endemic).

Classification. Troglophile.

IUCN RedList. NT.

Records in Greek caves. Aegean: Thasos: Drakotrypa Cave (Riedel 1988b, 1992, 1996; Beron 2016; Paragamian et al. 2023; present study).

***Balcanodiscus frivaldskyanus* (Rossmässler, 1842)**

Distribution. Eastern Balkan.
Classification. Troglophile.
IUCN RedList. LC.
Records in Greek caves. Thraki: Evros: Koufovouno Cave (Riedel 1969, 1985b, 1988b, 1992, 1996; Beron et al. 2004; Beron 2016; Paragamian et al. 2023; present study).

***Balcanodiscus* sp.**

Records in Greek caves. Makedonia: Serres: Alistrati Cave (Beron 2016; Paragamian et al. 2023).

Genus *Doraegopis*

***Doraegopis subaii* Riedel, 1990**

Distribution. Greek endemic, Sterea Ellada (stenoendemic).
Classification. Troglophile.
IUCN RedList. VU.
Records in Greek caves. Sterea Ellada: Fthiotida: Mavri Troupa Cave (Bank and Menkhorst 1988; Riedel 1990, 1992, 1996; Beron 2016; Paragamian et al. 2023; present study).

***Doraegopis* sp.**

Records in Greek caves. Peloponnisos: Achaia: Limnon Cave (Riedel 1982, 1986, 1992; Bank and Menkhorst 1988; Beron 2016; Paragamian et al. 2023).

Genus *Zonites*

***Zonites casius* Martens, 1889**

Distribution. Aegean Islands-Opposite Turkish mainland.
Classification. Trogloxene.
IUCN RedList. NT.
Records in Greek caves. Aegean: Kasos: Stylokamara Cave (Riedel 1985a).

***Zonites nikariae* Pfeffer, 1930**

Distribution. Greek endemic, Aegean Islands.

Classification. Troglophile.
IUCN RedList. VU.
Records in Greek caves. Aegean: Ikaria: Foutra Raos Cave (Martens 1889; Riedel 1985a, 1992; Beron 2016; Paragamian et al. 2023).

Among the 113 species, 15 were not identified at the species level, i.e. *Pagodulina* sp., and were therefore excluded from any cave-dwelling category. The remaining 98 species include 14 troglobionts (14%), 39 troglophiles (40%) and 45 (46%) trogloxenes (Fig. 3). The percentage of troglobiont gastropods is lower than the total percentage of troglobionts in the cave fauna in Greece (22.2%) (Paragamian et al. 2023). The troglobiont gastropods belong to eight genera, namely *Balcanodiscus* (1 species) (Zonitidae), *Gyralina* (2 species), *Lindbergia* (4 species), *Vitrea* (1species) (Pristilomatidae), *Mediterranea* (1 species) (Oxychilidae), *Pholeoteras* (1 species) (Cyclophoridae), *Sciocochlea* (2 species), *Tsoukatosia* (1 species) (Clausiliidae) and *Speleodentorcula* (1 species—monotypic genus) (Argnidae). All but two species (*Pholeoteras euthrix* and *Tsoukatosia christinae*) are endemic to Greece and are listed as Threatened or Near Threatened in the IUCN Red List (2023) but none is legally protected. It is interesting to note that five of the endemic species are distributed on the mainland while the remaining eight are found on islands (Fig. 4). In particular, Corfu, an Ionian island, hosts four troglobiont species belonging to four genera, three of which are endemic to the island.

Almost half of the cave-dwelling gastropod species (49%), are endemic to Greece, which is close to the endemism percentage of the entire cave fauna of Greece, which is 45% (Paragamian et al. 2023). Many of these have a very restricted distribution, with twelve species being single-island endemics, and seven being single-cave endemics (Fig. 5).

The island of Crete is the most studied region of Greece, containing 55% of the explored caves. Mainland Greece and most of the Aegean and Ionian islands have not been intensively explored (Fig. 1). The reason for this difference in between regions is mostly a matter of sampling bias rather than the presence or absence of caves.

The maximum number of species reported in a cave—nine species—is recorded in two caves: Gerani Cave in Crete and Megalos Votsos Panagias Pothole in Fournoi. The majority of the caves (101) are reported to host only one species while 60 caves host 2–3 species and the remaining 19 host 4–7 species (Fig. 6).

Discussion and conclusions

In this study, 113 terrestrial gastropod species are reported from 182 Greek caves, thereby doubling the number of known cave-dwelling species and providing first records for 113 caves. The species belong to 49 genera within 23 families. The most species- and genus-rich families are Oxychilidae (19 species, 7 genera), Pristilomatidae (19 species, 3 genera), Clausiliidae (16 species, 6 genera) and Zonitidae (8 species, 4 genera). These, with the exception of Clausiliidae, are the most diverse subterranean families worldwide (Grego 2022) while all of them are among the most species-rich families in Greece with Clausillidae being the most speciose family in Greece (Vardinoyannis et al. 2018).

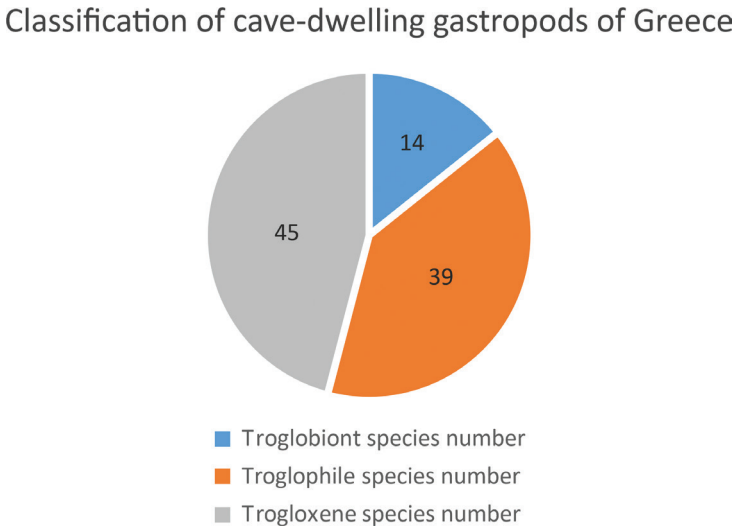

Figure 3. Ecological classification of the cave-dwelling gastropods in Greek caves.

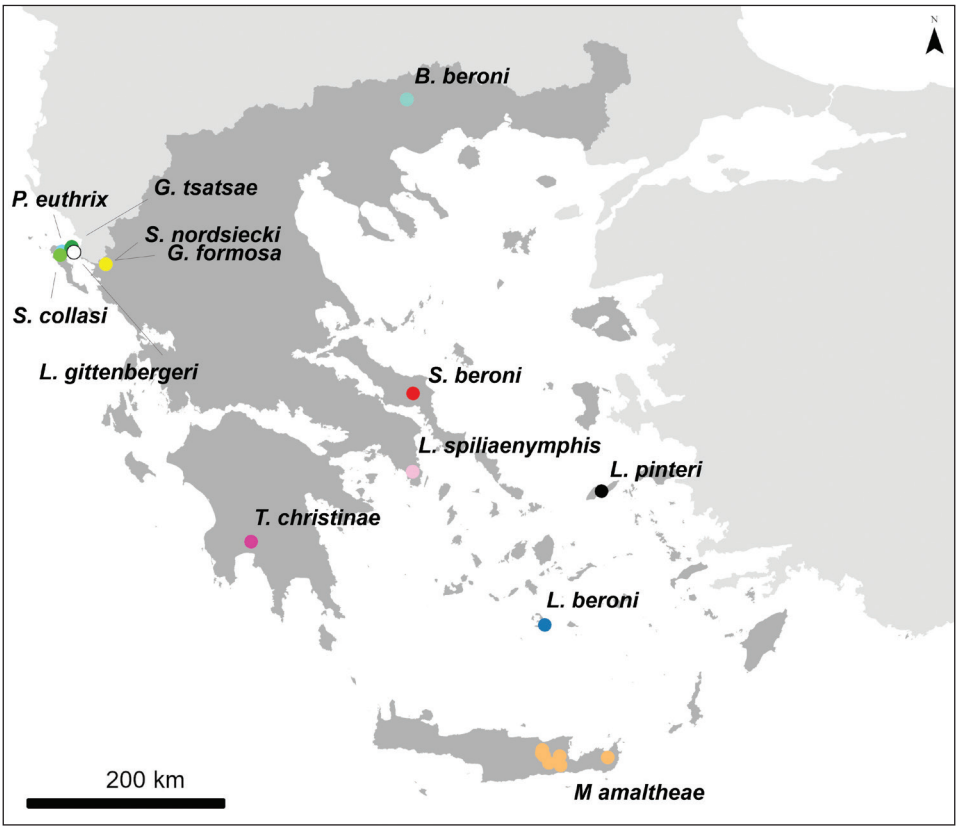

Figure 4. Troglobiont gastropod species of Greece.

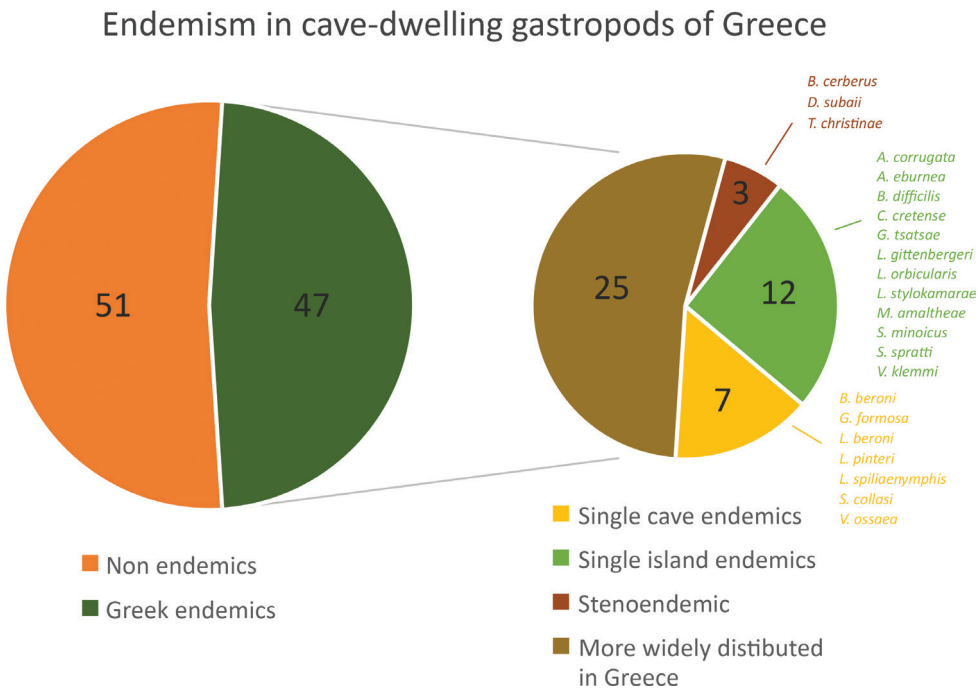

Figure 5. Number of endemic and non-endemic cave-dwelling gastropods in Greek caves.

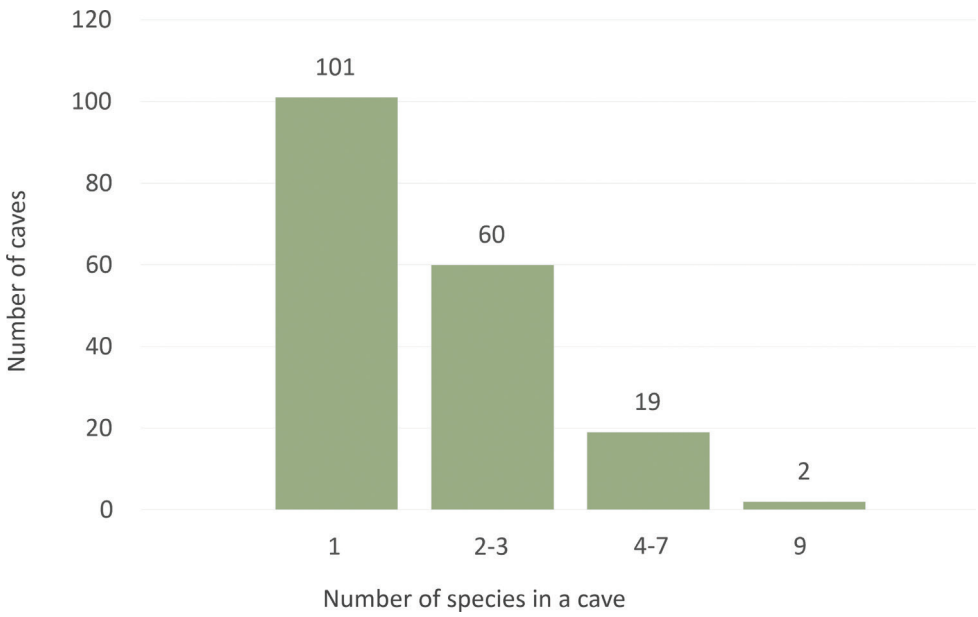

Figure 6. The number of caves in Greece that are reported to host from one to nine cave-dwelling gastropods.

Troglobionts and troglophiles comprise 14% and 40% of the cave-dwelling gastropod fauna respectively while 46% is characterized as trogloxene. Note that for a high proportion of taxa, the ecological classification cannot be made with certainty, regardless the chosen classification system (Sket 2004). For this reason, the reader should be cautious with these numbers. For example, many species classified as trogloxenes may have a variety of reasons penetrating caves such as seeking for more stable climatic conditions or—for the carnivorous species—searching for food (Weigand 2014). Most of the trogloxenes however, probably have an accidental presence in the caves. Many of these are not active visitors or by chance crawling in the caves but could have been washed into the cave by the rain. This is especially true for the species collected only as empty shells.

To date, more than 322 terrestrial gastropod species have been reported from subterranean habitats around the world, classified into 74 genera within 32 families (Grego 2022). The highest diversity of underground terrestrial gastropods is known from Europe (accounting for 59% of global taxa) and includes the Dinaric region (accounting for 25% of the global taxa) (Grego 2022). According to Sket et al. (2004), 49 species of obligate subterranean terrestrial gastropods have been reported from the Balkans. Thirty-six species are known from the western Balkans (Slovenia, Croatia, Bosnia and Herzegovina, Montenegro, Serbia, and North Macedonia) and 13 species from Greece (including the Aegean islands and Crete). Since then, more species have been described, e.g. at least seven species of the genus *Zospeum* [*Z. bucculentum*, *Z. clathratum*, *Z. manitaense*, *Z. pagodulum*, *Z. robustum and Z. simplex* all described by Inäbnit, Jochum & Neubert, 2019 in Inäbnit et al. (2019) and *Z. tholussum* Weigand, 2013 (Weigand 2013)].

It is difficult to compare the fauna of Greece with other countries, mainly the neighboring ones. One reason for this is that caves are generally underexplored (Wynne 2022). Even if a cave has been explored for its fauna, the data sometimes depend on the specialty of the researcher. For example, a bat specialist, will in general focus on bats and not on snails while a malacologist may not focus on or collect arthropods. Another reason is that the data is not easily accessible and there are not lists for every region and/or country. Finally, it is difficult to define the exact number of troglobiontic taxa of terrestrial gastropods, due to different definitions of the term "troglobiont", to inadequate knowledge of the morphology and biology of the taxa, and undefined taxonomic status (Gottstein 2002). Focusing on the Balkans, aggregated data are available for Bulgaria and Croatia. Although the latter is not a direct neighbour of Greece, it is worth mentioning because the karst areas in Croatia, are part of the Dinarides and most part of Greece, is an extension of them. The Dinarides, have been defined as a global hotspot for subterranean biodiversity (Gottstein 2002; Grego 2022; Lukić et al. 2023). In Croatia (56,600 km^2, more than 7,000 registered caves and potholes), although there has been no systematic research on terrestrial cave malacofauna, at least 19 troglobiont molluscan species have been recorded, seven of which are endemic to Croatia and one species common to Greece, *Pholeoteras euthrix* (Gottstein 2002). In total, two genera are common between the two countries (*Pholeoteras* and *Gyralina*).

On the other hand, Bulgaria (111,000 km^2, 5,000 caves and potholes) and Greece, two neighbouring countries that share the speleozoogeographic zones of Rhodopes (Georgiev 1977 in: Beron 2001), do not share any troglobiont species. According to Beron (2001), of the 20 terrestrial mollusc species reported from the 250 researched Bulgarian caves, none are considered troglobionts.

Of the 10,000 known caves of Greece, only 558 have been speleobiologically explored, while snails have been recorded in 182. Although speleobiologists have done a lot of work in the recent decades, there are still many more caves to be surveyed. The case of *Mediterranea amaltheae*, which was considered a single-cave endemic and in the present study was found in six more caves on the island of Crete, is evidence that the caves of Greece are undersuveyed. The new generation of speleobiologists in Greece, still have a broad field to explore. We believe that with the increasing exploration of Greek caves, the number of known species inhabiting caves will increase and new species will be discovered.

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Supplementary material 1

List of caves and their locality. Caves with an asterisk (*) are artificial caves.

Authors: Danae Karakasi

Data type: xlsx

Explanation note: The table contains all caves mentioned in the text with detailed geographic information.

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A new species of *Stygobromus* Cope, 1872 (Amphipoda, Crangonyctidae) from a hypotelminorheic seepage spring in Washington, D.C., USA

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Abstract

We describe a new species of subterranean amphipod (Amphipoda: Crangonyctidae) in the genus *Stygobromus* from a hypotelminorheic seepage spring at Shepherd Parkway, part of National Capital East Parks, Washington, D.C., USA, part of the National Park System, using both morphological and genetic approaches. The Anacostia Groundwater Amphipod, *S. anacostensis* **sp. nov.** is a member of the *S. tenuis* species group but differs from related congeners based on body size, serrate blade-like edge of both palms of gnathopods 1 and 2, presence of rastellate setae on the posterodistal margin of the carpus of gnathopod 2, and aspects of the second antennae, mandibular palp, pereopods 5–7, uropods 1 and 2, and telson. Moreover, *S. anacostensis* **sp. nov.** is genetically distinct from *S. tenuis* in the Washington D.C. metropolitan area. The description of *S. anacostensis* **sp. nov.** increases the number of described *Stygobromus* species to eight in the Washington D.C. area and highlights the need for continued biodiversity studies, even in regions that have received considerable attention.

Keywords

Amphipod, crustacean, District of Columbia, groundwater, species delimitation, stygobite, stygobiont, subterranean

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Introduction

The Holarctic amphipod genus *Stygobromus* Cope, 1872 is comprised of some 137 described stygobiotic (obligate subterranean) species and several undescribed forms mentioned in the literature, with greatest diversity in the Nearctic (Holsinger 1967, 1974, 1978; Holsinger and Sawicki 2016; Cannizzaro et al. 2019). *Stygobromus* diversity is high in groundwater habitats of the Piedmont and Atlantic Coastal Plain of Maryland, Virginia, and the District of Columbia, from which 13 species have been described, respectively (Holsinger 2009; Holsinger et al. 2011; Culver et al. 2012). *Stygobromus* are extraordinarily diverse in hypotelminorheic habitats and associated seepage springs, a shallow subterranean habitat (SSH; Culver et al. 2006; Culver and Pipan 2011, 2014; Pipan et al. 2012) in the lower Potomac River Basin in and near the Washington D.C. metropolitan area where seven species have been documented (Feller 1997; Hobson 1997; Culver and Šereg 2004; Holsinger 2009; Pipan et al. 2012).

Over 150 seepage springs have been identified in the Washington D.C. metropolitan area (Hutchins and Culver 2008; Culver et al. 2012; Keany 2016; Keany et al. 2019). The study and collection of groundwater fauna from these springs continues to improve our understanding on the distribution and ecology of *Stygobromus* spp. and uncover new diversity. Moreover, cryptic genetic variation and diversity is a common finding of phylogenetic studies of subterranean fauna (Zakšek et al. 2009; Niemiller et al. 2012; Hedin 2015), including amphipods (Lefebure et al. 2006; Finston et al. 2007; Bradford et al. 2010; Delic et al. 2017). Niemiller et al. (2018) discovered substantial genetic variation up to 13.7% uncorrected sequence divergence at the mitochondrial cytochrome oxidase subunit 1 (*co1*) locus among populations of the *S. tenuis* species group in the Atlantic Coastal Plain of Virginia and Washington, D.C., indicating the strong potential for cryptic diversity.

Here we describe *S. anacostensis* sp. nov. from a hypotelminorheic seepage spring at Anacostia Park in metropolitan Washington, District of Columbia based on morphological examination and genetic analyses of five loci commonly used in phylogenetic studies of amphipods (e.g., Englisch and Koenemann 2001; Hou et al. 2007, 2011; Kornobis et al. 2011).

Materials and methods

Collection site and approach

Hypotelminorheic habitats and associated seepage springs are shallow subterranean habitats, characterized by small flows of water in slight depressions lined with decaying leaves (Culver et al. 2006; 2012). Seepage springs drain a small area, often less than a hectare, and the habitat only reaches a few meters in depth. The specimens were collected as part of a census of seepage springs in National Capital East (NACE), a unit of the National Park Service. Over 150 seeps were discovered during

this census (Keany et al. 2018). Specimens were preserved in 100% ethanol and stored at -20 °C for molecular analysis. Specimens examined were deposited in the Smithsonian National Museum of Natural History Invertebrate Zoology Collection in Washington, D.C.

Morphological analyses

To enhance the ability to clearly perceive suture lines and setation patterns, prior to dissection, most specimens were digested overnight in 400 µl of Zymo Research 2× digestion buffer, 40 µl of proteinase K and 360 µl of molecular grade water at 37 °C. Specimens were then stained by being placed into a 2% Lignin Pink solution for at least 2 hours. Specimens were dissected using a Leica M125 stereomicroscope (Leica, Wetzlar, Germany). Slide preparations were made by mounting dissected appendages and other body parts in glycerin. These temporary slide mounts were then examined, and drawings of pertinent structures were prepared using a Leica DM 1000 compound microscope outfitted with a drawing tube. Illustrations were finalized for publication in Adobe Illustrator CC. ImageJ software (Abramoff et al. 2004) was used for body length and appendage measurements. Body length was measured as the distance from the rostrum to the base of the telson following the contour of the body. Dissected parts were later transferred to small vials of ethanol for storage and/or future study.

Nomenclature for setal patterns on the third article of the mandibular palps follow Karaman (1969). The following terms are used. "Defining angle" refers to the posterior margin of the palm and the distalmost point of the posterior margin of the propodus, the area where the tip of the dactylus closes on the propodus; and "clothes-pin setae" refers to two notched robust setae present on the basal segments of the pleopod inner rami as illustrated in Holsinger (2009).

DNA extraction, amplification, and sequencing

Genomic DNA was extracted for select specimens of *S. anacostensis* sp. nov. and other members of the *S. tenuis* species group in the Washington, D.C. area (Table 1) using the Qiagen DNeasy® Blood and Tissue Kit (Qiagen, Germantown, Maryland, USA) following the manufacturer's protocol. We amplified using polymerase chain reaction (PCR) fragments of five loci: 535-bp of mitochondrial cytochrome oxidase subunit 1 (*co1*), 428-bp of mitochondrial 16S rDNA (*16s*), 329-bp of nuclear histone h3 (*h3*), 611-bp of nuclear 18S rDNA and 835-bp of nuclear 28S rDNA (*28s*). PCR primers used in this study are presented in Table 2.

PCR products were purified using ExoSAP-IT (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and sequenced in both directions using BigDye chemistry at Eurofins Genomics (Louisville, Kentucky, USA). Low quality reads at the ends of forward and reverse sequences were trimmed and ambiguous base calls verified manually by examining electropherograms. Sequences were assembled into contigs using Chromas

Table 1. *Stygobromus* populations sampled in the Washington, D.C., USA area and GenBank accession numbers for five loci used in the current study. Additional sequences downloaded from GenBank are also included.

Sample	n	Species	State	County/ District	Locality	*co1*	*16s*	*18s*	*28s*	*h3*
SP94	8	*Stygobromus anacostensis*	DC	Washington	Malcolm X Ave seep	OR506977–OR506984	OR530101–OR530107	OR530128–OR530135	OR530153–OR530160	OR506374–OR506381
SP100	3	*Stygobromus anacostensis*	DC	Washington	Malcolm X Ave seep	OR506974–OR506976	OR530108–OR530110	OR530136–OR530138	OR530161–OR530163	OR506372–OR506373
SP31	2	*Stygobromus allegheniensis*	WV	Berkeley	Caskey Spring	KY748254	OR530092	OR530121	OR530142	OR506362
SP26	3	*Stygobromus t. potomacus*	VA	Arlington	Pimmit Run Seepage Spring A	KY748251, OR506961–OR506962	OR530090–OR530091	OR530119–OR530120	OR530145–OR530146	OR506363–OR506364
SP80	3	*Stygobromus t. potomacus*	DC	Washington	Upper Kennedy Street Spring, Rock Creek Park	KY748252, OR506963–OR506964	OR530093–OR530094	OR530122–OR530123	OR530149–OR530150	OR506360–OR506361
SP86	3	*Stygobromus hayi*	DC	Washington	West Rapids Spring, Rock Creek Park	KY748253, OR506965–OR506966	OR530099–OR530100	OR530126–OR530127	OR530143–OR530144	OR506358–OR506359
SP101	1	*Stygobromus t. potomacus*	DC	Washington	Shepherd Park seep	OR506971	–	OR530139	–	OR506371
SP102	2	*Stygobromus t. potomacus*	VA	Arlington	Pimmit Run Seepage Spring B	OR506972–OR506973	OR530097–OR530098	OR530140–OR530141	OR530147–OR530148	OR506369–OR506370
SP104	2	*Stygobromus t. potomacus*	VA	King George	Caledon State Park, Site 4	OR506969–OR506970	OR530111–OR530112	OR530115–OR53016	OR530164–OR530165	OR506367–OR506368
SP105	2	*Stygobromus t. potomacus*	VA	King George	Caledon State Park, Site 1	OR506967–OR506968	OR530113–OR530114	OR530117–OR530118	OR530166–OR530167	OR506365–OR506366
SP95	2	*Stygobromus t tenuis*	MD	Harford	Wilkenson Road seep, Susquehana State Park	OR506985–OR506986	OR530095–OR530096	OR530124–OR530125	OR530151–OR530152	OR506356–OR506357
	1	*Stygobromus t. potomacus*	VA	Caroline	Fort A.P. Hill	KU869712	KU869712	–	–	–
	1	*Stygobromus tenuis*	VA	Caroline	Goldenvale Creek	KP693695	–	–	–	–
	1	*Stygobromus allegheniensis*	NY	Ulster	Xanadu Cave, Mohonk Preserve	–	–	–	–	KP696362
	1	*Stygobromus allegheniensis*	NY	Albany	Clarksville Cave	–	–	–	–	KP696363
	1	*Stygobromus allegheniensis*	NY	Ulster	Ice Cave no. 1, Minnewaska State Park Preserve	–	–	–	–	KP696361

Table 2. Loci and associated PCR primers to infer phylogenetic relationships of *Stygobromus* in the current study.

Locus	Name	Genome	Length	Primers	Reference(s)
co1	cytochrome oxidase subunit 1	mtDNA	535	*jgLCO1490* – TITCIACIAAYCAYAARGAYATTGG *jgHCO2198* – TAIACYTCIGGRTGICCRAARAAYCA	Geller et al. (2013)
16s	16S ribosomal DNA	mtDNA	428	*16STf* – GGTAWHYTRACYGTGCTAAG *16Sbr* – CCGGTTTGAACTCAGATCATGT	Palumbi et al. (1991), Macdonald et al. (2005)
18s	18S ribosomal DNA	nuclear	611	*18Sf* – CCTAYCTGGTTGATCCTGCCAGT *18S700r* – CGCGGCTGCTGGCACCAGAC	Englisch and Koenemann (2001)
28s	28S ribosomal DNA	nuclear	835	*28Sf* – TTAGTAGGGGCGACCGAACAGGGAT *28S1000r* – GACCGATGGGCTTGGACTTTACACC	Hou et al. (2007)
h3	histone H3	nuclear	329	*H3f* – AAATAGCYCGTACYAAGCAGAC *H3r* – ATTGAATRTCYTTGGGCATGAT	Corrigan et al. (2014)

v2.6.6 (Technelysium, South Brisbane, Queensland, Australia), then aligned using MUSCLE (Edgar 2004) in the program MEGA v.7.0.26 (Kumar et al. 2016). All new sequences generated during this study were accessioned into GenBank (Table 1). We also included additional sequences available for the *S. tenuis* species group on GenBank accessioned from previous studies (e.g., Aunins et al. 2016; Niemiller et al. 2018; Benito et al. 2021; Table 1).

Phylogenetic analyses

Uncorrected p-distances for each locus were calculated in MEGA. Optimal models of nucleotide substitution for each locus, including first, second, and third codon positions for *co1*, were determined in jModelTest2 (Darriba et al. 2012) using corrected Akaike's Information Criterion (AICc). Five molecular datasets were assessed: *co1*, *16s*, mtDNA (*co1+16s*), nucDNA (*18s+28s+h3*), mtDNA+nucDNA (*co1+16s+18s+28s+h3*). Maximum likelihood (ML) analyses were conducted in RAxML v.8 (Stamatakis 2014). A consensus tree was generated for each dataset using rapid bootstraps for 1,000 replicates under a GTR+Γ model of evolution. Bayesian inference (BI) analyses were conducted in MrBayes v.3.2.6 (Ronquist et al. 2012) using a random starting tree with three heated and one cold chain under a temperature profile of 0.2. BI analyses were run independently twice for 50,000,000 generations and sampled every 1,000 generations under the models of evolution determined by jModelTest2. Stationarity was determined by examining the average standard deviation, assuming stationarity was achieved if the average standard deviation was < 0.005. In general, the first 12.5 million generations (25%) were discarded as burn-in. Convergence of runs was assessed utilizing Tracer v. 1.4 (Rambaut and Drummond 2007). The remaining trees from the stationarity distribution were sampled to generate a 50% majority-rule consensus tree.

Haplotype networks for nuclear loci were constructed using the median-joining network algorithm (Bandelt et al. 1999) using the program PopART v1.7 (Leigh and Bryant 2015).

Species delimitation

We employed three species delimitation approaches on the mtDNA dataset to define molecular operational taxonomic units (MOTUs): Automatic Barcode Gap Discovery (ABGD; Puillandre et al. 2012) and Poisson Tree Processes (PTP; Zhang et al. 2013), and Multi-rate Poisson Tree Processes (mPTP; Kapli et al. 2017). ABGD partitions sequences into candidate species based on a statistically inferred barcode gap defined as a significant disparity between pairwise genetic distances, presumably between intraspecific and interspecific distances. This process is applied recursively to newly obtained groupings of sequences to assess the potential of internal division. This method was employed excluding outgroup taxa via the ABGD web server (http://wwwabi.snv.jusieu.fr/public/abgd/abgdweb.html) using the Kimura two-parameter (Kimura 1980) model with a standard X (relative gap width) = 1.5. The initial development of the multispecies coalescent PTP model assumed one exponential distribution for speciation events and one for all coalescent events (Zhang et al. 2013). The mPTP approach fits speciation events for candidate species to a unique exponential distribution (Kapli et al. 2017) rather than assuming one exponential distribution for speciation events and one for all coalescent events in PTP models (Zhang et al. 2013). Both the PTP and mPTP methods were employed using rooted ML trees for each dataset for 10 million generations, with a burn-in discarding the first 25% in mptp (Kapli et al. 2017).

Conservation assessment

We conducted IUCN Red List and NatureServe conservation assessments following IUCN (2001) and Master et al. (2009). Both assessments rank taxa into one of seven unique categories on a continuum of increasing extinction risk. Risk categories were calculated using the RAMAS Red List 3.0 (Akcakaya et al. 2007) and the NatureServe Rank Calculator v3.186 (Faber-Langendoen et al. 2012) for the IUCN Red List and NatureServe assessments, respectively. Geographic range size was calculated using two different measures for the extent of occurrence (EOO) and area of occupancy (AOO).

Results

Class Crustacea Brünnich, 1772
Order Amphipoda Latreille, 1816
Infraorder Gammarida Latreille, 1802
Superfamily Crangonyctoidea Bousfield, 1973

Family Crangonyctidae Bousfield, 1973; emended by Holsinger, 1977
Genus *Stygobromus* Cope, 1872

***Stygobromus anacostensis* Cannizzaro, Sawicki, & Niemiller, sp. nov.**
https://zoobank.org/D66AA3F8-A53B-41A2-B16C-CA9486A39BC1
Figs 1–8

Type material. *Holotype*: male 5.9 mm, from USA, Washington, District of Columbia, Anacostia Park, (38.83059°N, -76.9995°W), deposited in the collection of the United States National Museum of Natural History, Smithsonian Institution, Washington, D.C (USNM 1606902); female allotype 5.3 mm (USNM 1606903). Holotype male and female allotype collected 18 October 2021 by Lizzy Sartain.

***Paratypes*:** 1 male (USNM 1606904) and 2 females (USNM 1606905–1606906) collected on 18 October 2021 by Lizzy Sartain from type locality; 1 female collected on 20 September 2021 by Lizzy Sartain from the type locality (USNM 1606907).

Etymology. The specific epithet *anacostensis* refers to its occurrence in Anacostia neighborhood in Washington, D.C., USA. It is part of the Anacostia River drainage.

Type locality. USA. Washington, District of Columbia, hypotelminorheic seepage spring in a highly urbanized area that emerges from a small, 2-m high rockface ca. 5 m from Malcolm X Avenue SE in Shepherd Parkway (Figs 9, 10, 38.83059°N, -76.9995°W). Shepherd Parkway is part of National Capital Parks East. Most individuals were collected in the water flowing over moss-covered rocks. A few individuals were also present in decaying leaves at the base, a more usual hypotelminorheic habitat (Culver et al. 2006, 2012). The site is at the extreme tip of Shepherd Parkway, a unit of National Capital East (National Park Service). The width of park land is about 20 m and is bordered by Malcolm X Avenue. The site was discovered when a park ranger noticed extensive ice on the adjacent sidewalk resulting from flow from the seep.

Diagnosis. Small stygomorphic species distinguished from other members of the *tenuis* group by size, largest male 5.9 mm, largest female 5.3 mm and as follows: *S. tenuis tenuis* – by antenna 2 subequal to or shorter than antenna 1; *S. tenuis potomacus* – only 2 C-setae on mandibular palp and up to 8 E-setae; *S. allegheniensis* – pereopods 5–7 basis posterior margin weakly convex, and telson tapering distally; *S. hayi* – by significantly less spinose uropods 1 and 2, and telson with significantly fewer apical robust setae. Further distinguished from all *tenuis* group species by gnathopods 1 and 2 with a serrate blade-like edge running the length of both palms, and by the postero-distal margin of gnathopod 2 carpus possessing rastellate seta(e).

Description. Male: holotype, USNM 1606902 (Fig. 1A); Size 5.9 mm.

***Antennae*.** Antenna 1 (Fig. 2A): 45% body length, 60% length of antenna 2 (in paratype (USNM 1606904); primary flagellum with 18 segments, aesthetascs on most segments, aesthetascs shorter than respective segments; accessory flagellum 2-segmented, reaching beyond first segment in length.

Antenna 2 (Fig. 2B): damaged in holotype, description based on paratype (USNM 1606904); gland cone distinct; peduncle 80% length of flagellum, with weak plumose

Figure 1. *Stygobromus anacostensis* sp. nov., habitus: **A** holotype male, 5.9 mm (USNM 1606902) **B** Allotype female, 5.3 mm (USNM 1606903). Scale bar: 1 mm.

setules concentrated on postero and anterodistal margins of segments 4 and 5, peduncle segment 4 subequal in length to 5; flagellum 12-segmented, segment 5 with robust seta on anterodistal margin and segments 6 and 7 with robust seta placed along posterodistal margins, small calceoli-like structures apically on flagellar segments 5–12.

Mouthparts (Figs 2C, D, 3). Mandibles: left mandible (Fig. 2C) incisor 5-dentate, lacinia mobilis 5-dentate, with 7 robust serrate and numerous plumose accessory setae; molar process reduced with simple seta; palp with 3 segments, second segment 85% length of third, with inner margin bearing 8 setae and sparse fine setae; segment 3 with 2 C-setae, 5 E-setae, 1 B-seta, and 8 plumose D-setae, lacking A-setae; face of article covered in numerous, fine pubescent setae.

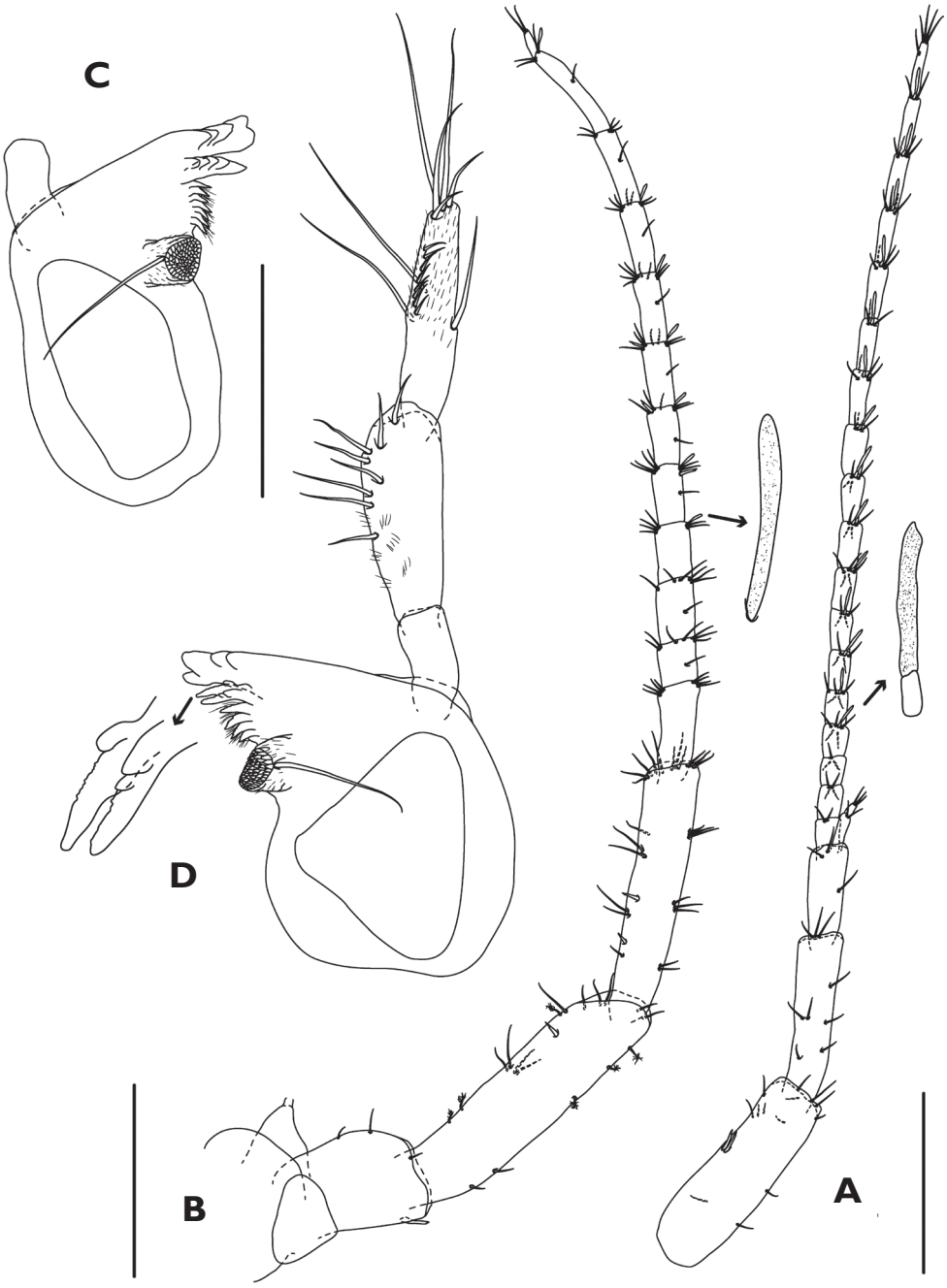

Figure 2. *Stygobromus anacostensis* sp. nov., Holotype male, 5.9 mm (USNM 1606902): **A** antenna 1 (single aesthetasc enlarged) **C** left mandible (palp omitted) **D** right mandible (lacinia mobilis enlarged). Paratype male, 5.7 mm (USNM 1606904): **B** antenna 2 (single calceolus enlarged). Scale bars: 0.5 mm (**A, B**); 0.25 mm (**C, D**).

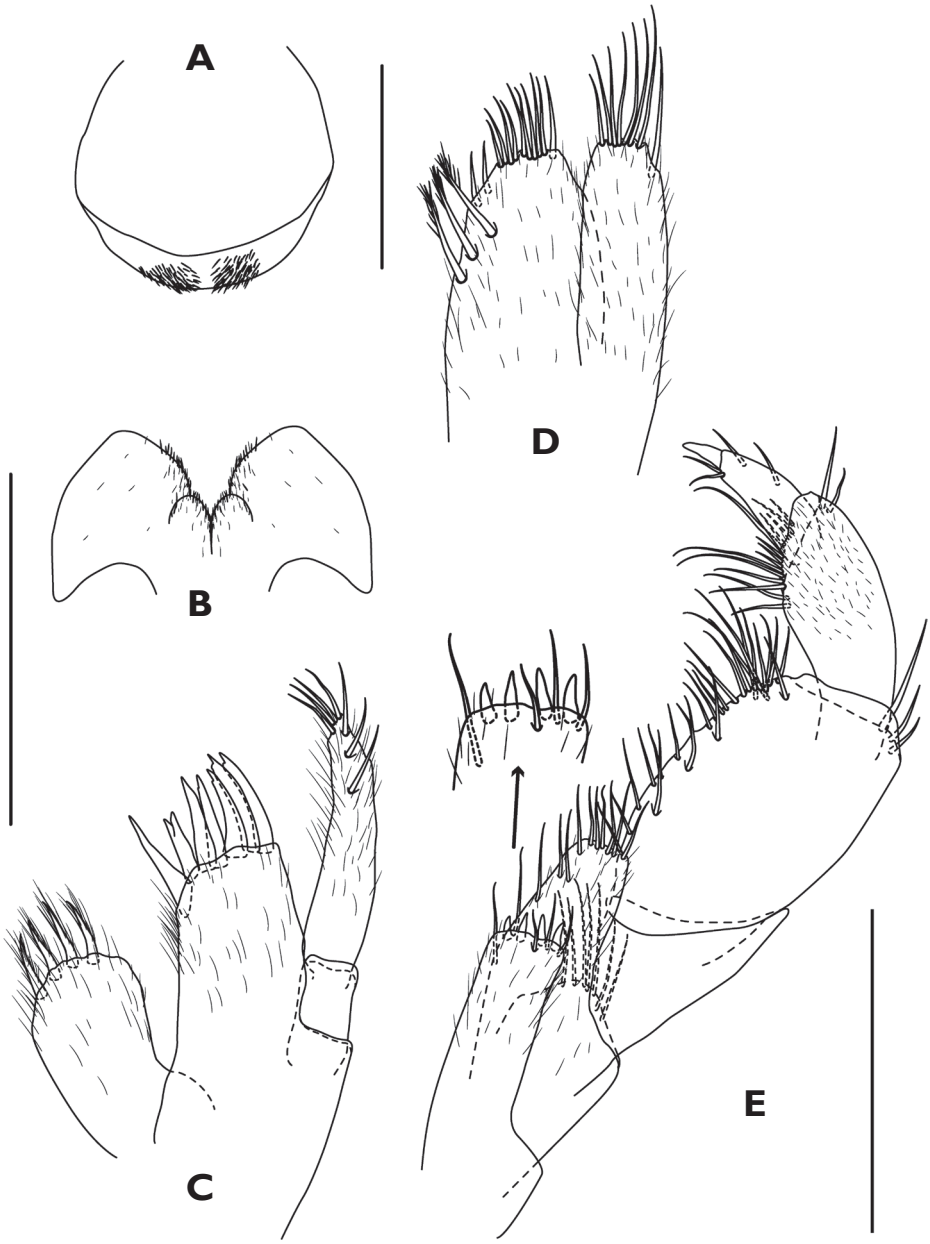

Figure 3. *Stygobromus anacostensis* sp. nov., Paratype male, 5.7 mm (USNM 1606904): **A** upper lip **D** maxilla 2. Holotype male, 5.9 mm (USNM 1606902): **B** lower lip **C** maxilla 1 **E** maxilliped (distal margin of inner plate enlarged). Scale bars: 0.25 mm.

Right Mandible (Fig. 2D): incisor 4-dentate, lacinia mobilis bifurcate, both lobes with numerous protuberances; accessory setae row with 4 robust, serrate setae and numerous plumose setae; molar process reduced with simple seta. Palp with 3 articles, relative articles length and setation patterns as in left mandible.

Upper Lip (Fig. 3A): rounded, apical margin of labrum with numerous fine setae. Lower Lip (Fig. 3B): inner lobes distinct; outer margin of outer lobe sparsely covered in fine setae; inner margin of outer lobe heavily setose.

Maxilla 1 (Fig. 3C): missing in holotype, description based on paratype (USNM 1606904); inner plate with 4 plumose marginal setae and numerous fine, pubescent setae covering entire plate; outer plate with 7 apical comb spines, pubescence covering inner margin, decreasing laterally and proximally; palp with 2 segments, distal segment covered in pubescence; subapical margin of distal article with 3 long setae, apical margin with 4 setae.

Maxilla 2 (Fig. 3D): missing in holotype, description based on paratype (USNM 1606904); both inner and outer plates covered in pubescent setae; outer plate not as wide as inner plate, not narrowing distally, with numerous distal setae; inner plate narrowing slightly distally, with numerous apical setae and 3 large plumose facial setae.

Maxilliped (Fig. 3E): inner plate shorter than outer plate, with 4 naked cuspidate setae 3 setae along apical margin, surface of plate covered in fine pubescence; outer plate armed with numerous setae covering inner and apical margins; palp second segment with numerous marginal setae, third article with numerous marginal/submarginal setae; dactyl with 2 outer setae and 2 inner setae.

Gnathopods. Gnathopod 1 (Fig. 4A): coxal plate with 3 apical setae; basis with long setae inserted along anterior, and posterior margins, small patch of pubescence on posterodistal corner; ischium with 4 setae and pubescence along posterior margin; merus weakly pubescent along posterior surface, numerous distal setae, and robust seta along anterior margin; carpus approximately 50% length of propodus with robust seta along anterior margin and a group of setae on anterodistal margin, one of which is approximately 50% length of propodus, posterior margin with single group of plumose setae and 6 submarginal setae directed distally; propodus 1.3× longer than broad, with 1 marginal anterior seta, 4 superior medial setae, with middle group of medial setae paired, 4 setae inserted at anterodistal corner, 6 inferior medial setae and numerous plumose posterior setae; palm oblique, concave, with serrate blade-like edge running the length, 7 outer and 7 inner bifid robust setae, 6 outer setae, and inner seta; inner margin of defining angle with 3 bifid robust setae, outer margin with 4 bifid robust setae; dactylus with outer seta and 7 short setae covering the entire inner margin and 3 setae placed along the inner margin at base of nail.

Gnathopod 2 (Fig. 4B): coxal plate with 4 apical setae and facial seta; basis with long setae inserted along anterior, and posterior margins, small patch of pubescence on posterodistal corner; ischium with 3 setae and pubescence along posterior margin; merus with pubescence covering posterior surface and 4 posterodistal setae and robust seta along anterior margin; carpus approximately 75% length of propodus, with robust seta along anterior margin and two setae on anterodistal margin, one of which is approximately 33% length of propodus, posterior margin with 4 groups of plumose setae, distal-most bearing 3 rastellate setae, and 3 submarginal setae directed distally; propodus 1.3× longer than broad, with marginal anterior seta, 5 superior medial setae, distal-most paired, 5 setae inserted at anterodistal corner, 5 inferior medial setae, proximal-most paired, and 8 groups of plumose setae along posterior margin; palm

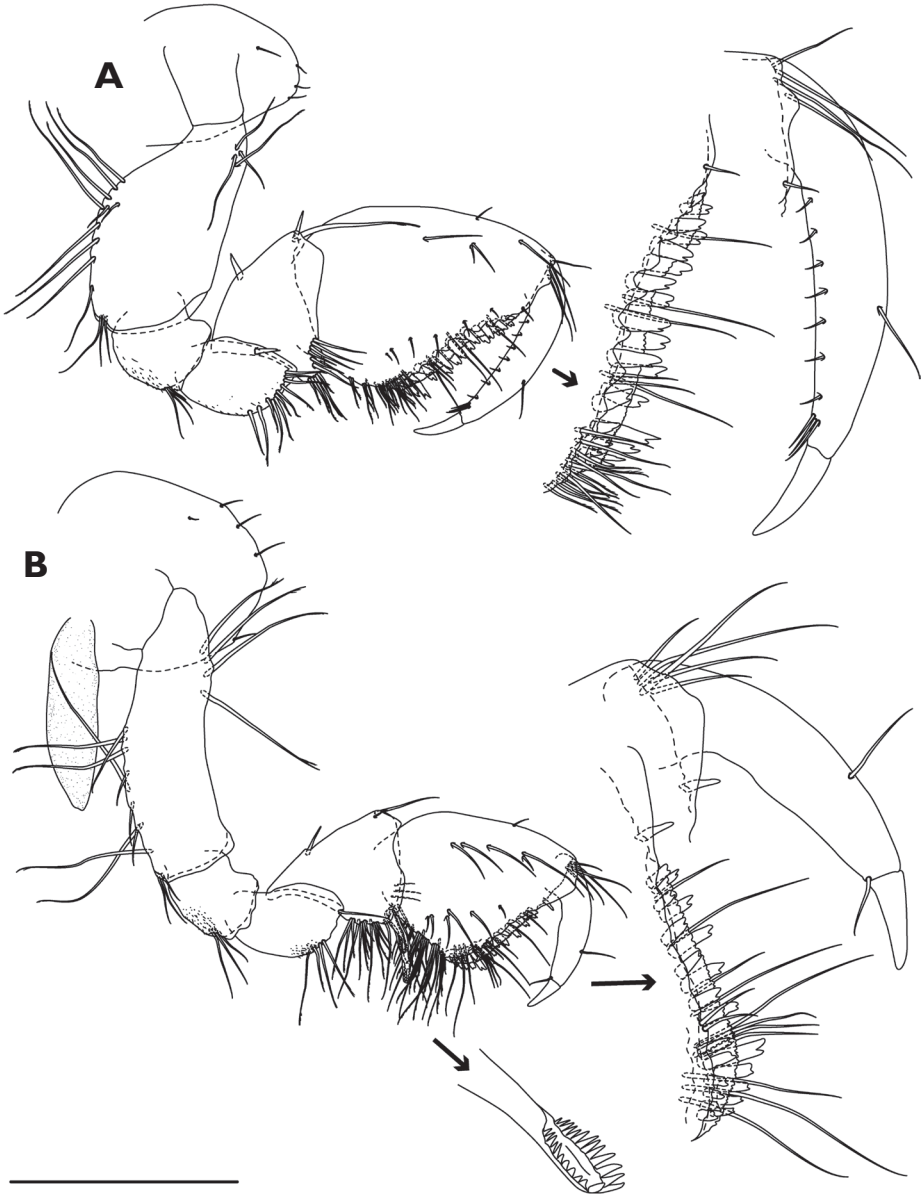

Figure 4. *Stygobromus anacostensis* sp. nov., Holotype male, 5.9 mm (USNM 1606902): **A** gnathopod 1 (palm and dactyl enlarged) **B** gnathopod 2 (rastellate seta, palm and dactyl enlarged). Scale bar: 0.5 mm.

oblique, straight, with serrate blade-like edge running the length, 5 outer and 5 inner bifid robust setae, 5 outer setae, and 2 inner setae; inner margin of defining angle with 6 bifid robust setae, outer margin with 5 bifid robust setae; dactylus with outer seta and seta placed along the inner margin at base of nail.

Pereopods. Pereopod 3 (Fig. 5A): coxal plate with 5 apical setae; merus 1.4× longer than carpus, carpus approximately 85% of propodus in length; dactylus approximately

50% length of propodus, with plumose seta on posterior margin, 2 setae along anterior margin followed by thin seta on medial margin.

Pereopod 4 (Fig. 5B): subequal to pereopod 3 in length; coxal plate armed with 4 anterior and 3 posterior apical setae; merus approximately 1.6× longer than carpus; carpus approximately 60% length of propodus; dactylus approximately 40% length of propodus, setation as in pereopod 3.

Pereopod 5 (Fig. 5C): coxal plate large, bilobate with distinct anterior and posterior lobes, posterior lobe with 4 robust setae on distal corner; basis posterior margin weakly convex with 9 shallow serrations, anterior margin with 6 split-tipped robust setae and 3 distal split-tipped setae; merus subequal in length to carpus; carpus subequal to propodus, dactylus approximately 50% length of propodus, setation as in pereopod 4.

Pereopod 6 (Fig. 5D): coxal plate bilobate, with weakly produced anterior lobe, posterior lobe bearing 2 robust apical setae; basis posterior margin weakly convex with 8 serrations, anterior margin with 5 split-tipped robust setae, and 3 robust setae at anterodistal corner; merus approximately 1.2× length of carpus; carpus approximately 90% of propodus in length, dactylus approximately 50% length of propodus, setation as in pereopod 5.

Pereopod 7 (Fig. 5E): coxal plate small, subtriangular, with 4 posterior setae; basis posterior margin weakly convex with 10 serrations and straight distal corner, anterior margin with 8 split-tipped robust setae, and 2 robust setae at anterodistal corner; merus subequal in length to carpus; carpus approximately 80% length of propodus, dactylus approximately 40% length of propodus, setation as in pereopods 5, 6.

Gills (Fig. 5F). coxal gills on somites 2–6, somites 6 and 7 with bifurcate sternal gills.

Pleon. Epimera (Fig. 6A): first epimeron ventral margin with robust seta, distoposterior corner rounded, posterior margin with 2 setae. Second epimeron ventral margin with 3 robust setae, distoposterior corner rounded, posterior margin with 2 setae. Third epimeron ventral margin with 3 robust setae, distoposterior corner rounded, posterior margin with 2 setae.

Pleopods: pleopod 1 (Fig. 6B) peduncle lacking setae, with 2 coupling hooks; outer, inner rami with 8 and 11 segments respectively, basal segment of outer ramus with clothes-pin setae. Pleopod 2 peduncle lacking setae, with 2 coupling hooks; outer, inner rami with 7, 11 segments respectively, basal segment of outer ramus with clothes-pin setae. Pleopod 3 outer, inner rami with 7, 7 segments respectively, basal segment of outer ramus with clothes-pin setae.

Urosome. Mostly bare, with sparse setae covering dorsal surface. Uropod 1 (Fig. 6C): peduncle 1.4× inner ramus in length, with 8 outer robust setae and inner robust seta(e), posteromedial margin with distinct protuberance approximately 20% of inner ramus in length, dorsal margin weakly serrate; outer ramus approximately 80% length of inner, with 2 inner and outer robust setae and 4 apical robust setae; inner ramus possessing 3 outer and two inner robust setae, and 5 apical robust setae.

Uropod 2 (Fig. 6D): peduncle subequal in length to inner ramus, with 2 outer robust setae and inner robust seta; outer ramus approximately 88% length of inner ramus without robust setae along the inner and outer margins, and 4 apical robust setae; inner ramus with 2 outer and 2 inner robust setae, with 5 apical robust setae.

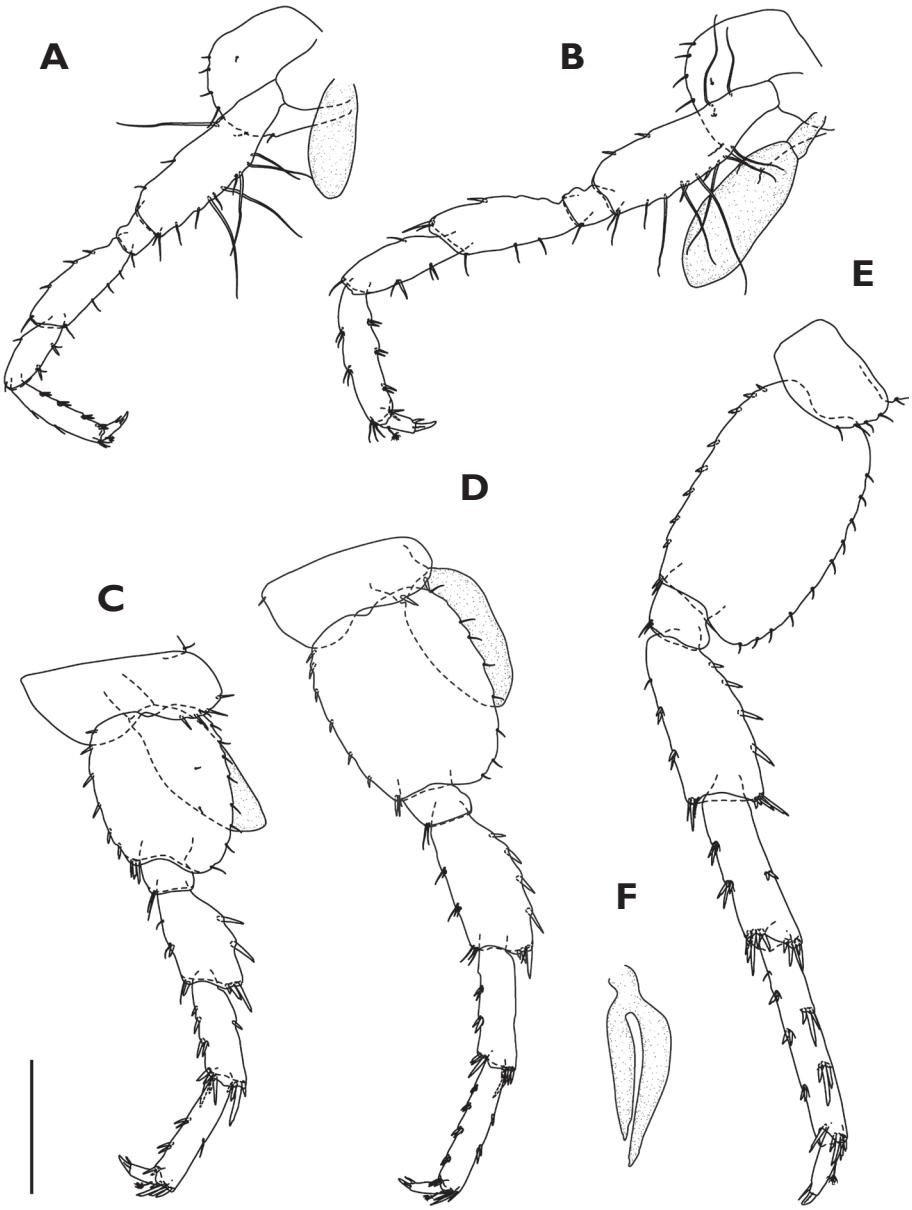

Figure 5. *Stygobromus anacostensis* sp. nov., Holotype male, 5.9 mm (USNM 1606902): **A** pereopod 3 **B** pereopod 4 **C** pereopod 5 **D** pereopod 6 **E** pereopod 7 **F** bifurcate sternal gill located on somites 6 and 7. Scale bar: 0.5 mm.

Uropod 3 (Fig. 6E): small, shorter than telson, uniramous; peduncle 2× length of ramus; ramus with 3 apical robust setae.

Telson (Fig. 6F). Telson entire, elongated, 1.5× longer than broad, weakly tapering distally; apex with 10 robust setae, and plumose seta, 2 plumose setae arise dorsolaterally from both outer margins.

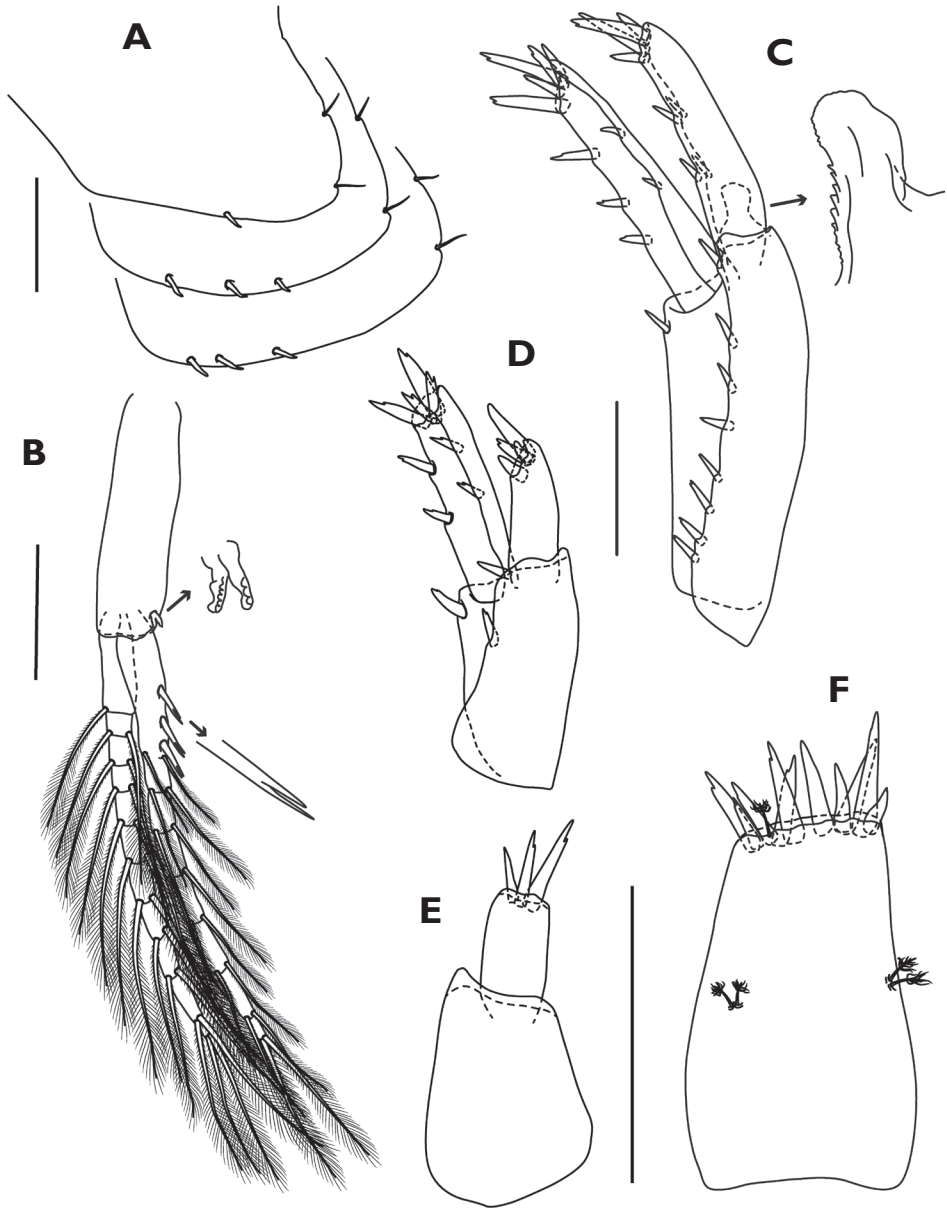

Figure 6. *Stygobromus anacostensis* sp. nov., Holotype male, 5.9 mm (USNM 1606902): **A** epimera 1–3 **B** pleopod 1 (coupling spines and clothes pin seta enlarged) **C** uropod 1 (posteromedial protuberance enlarged) **D** uropod 2 **E** uropod 3 **F** telson. Scale bars: 0.25 mm.

Female: allotype USNM 1606903 (Fig. 1B); Size 5.3 mm. Differing from male in several points, including, antennae; gnathopod shape and setation; uropods 1 and 2 shape and setation. Structures not described below are as in male.

Antennae. Antenna 1 (not illustrated, but see Fig. 1B): 50% body length, 1.5× longer than antenna 2; peduncle, flagellum lacking robust setae; primary flagellum

with 16 segments. Antenna 2 (Fig. 7A): gland cone distinct; peduncle 1.5× longer than flagellum, with robust setae anteriorly, laterally on segments 3, 4, peduncle segment 4 subequal in length to segment 5; flagellum 7-segmented, without small calceoli-like structures apically on distal flagellar segments.

Gnathopods. Gnathopod 1 (Fig. 7B): coxal plate with 3 apical and 2 facial setae; ischium with 2 setae and pubescence along posterior margin; carpus approximately 40% length of propodus with robust seta along anterior margin and a group of setae on anterodistal margin, one of which is approximately 50% length of propodus, posterior margin with single group of plumose setae and 4 submarginal setae directed distally; propodus 1.25× longer than broad, with 1 marginal anterior seta, 3 superior medial setae, 3 setae inserted at anterodistal corner, 3 inferior medial setae and numerous plumose posterior setae; palm oblique, straight, with serrate blade-like edge running the length, 5 outer and 5 inner bifid robust setae, 4 outer setae, and inner seta; inner margin of defining angle with 3 bifid robust setae, outer margin with 4 bifid robust setae; dactylus with outer seta and 4 short setae covering the inner margin and 2 setae placed along the inner margin at base of nail.

Gnathopod 2 (Fig. 7C): coxal plate with 6 apical setae and 2 facial setae; ischium with 2 setae and pubescence along posterior margin; merus with pubescence covering posterior surface and 4 posterodistal setae, without robust seta along anterior margin, and two robust setae along distal margin; carpus subequal in length to propodus, with robust seta along anterior margin and two setae on anterodistal margin, one of which is approximately 33% length of propodus, posterior margin with 3 groups of plumose setae, distal-most bearing rastellate seta, and 3 submarginal setae directed distally; propodus 1.1× longer than broad, with marginal anterior seta, 3 superior medial setae, 4 setae inserted at anterodistal corner, 4 inferior medial setae, and 5 groups of plumose setae along posterior margin; palm oblique, straight, with serrate blade-like edge running the length, 3 outer and 3 inner bifid robust setae, 3 outer setae, and 1 inner seta; inner margin of defining angle with 4 bifid robust setae, outer margin with 4 bifid robust setae; dactylus with outer seta and 4 short setae covering the inner margin and seta placed along the inner margin at base of nail.

Gills and brood plates. Gills as in male with coxal gills on somites 2–6, somites 6 and 7 with bifurcate sternal gills (Fig. 8A illustrates somite 7). Brood plates early in development in allotype, present on somites 2–5.

Urosome. Uropod 1 (Fig. 8B): peduncle 1.5× length of inner ramus, with 6 outer robust setae and inner robust seta(e), posteromedial margin lacking protuberance; outer ramus approximately 90% length of inner, with 1 inner and outer robust seta, and 4 apical robust setae; inner ramus possessing 2 outer and inner robust seta(e), and 5 apical robust setae.

Uropod 2 (Fig. 8C): peduncle subequal in length to inner ramus, with 2 outer robust setae and inner robust seta; outer ramus approximately 66% length of inner ramus with outer robust seta, and 4 apical robust setae, inner robust setae lacking.

Uropod 3 (Fig. 8D): small, shorter than telson, uniramous; peduncle 1.5× length of ramus; ramus with 4 apical robust setae.

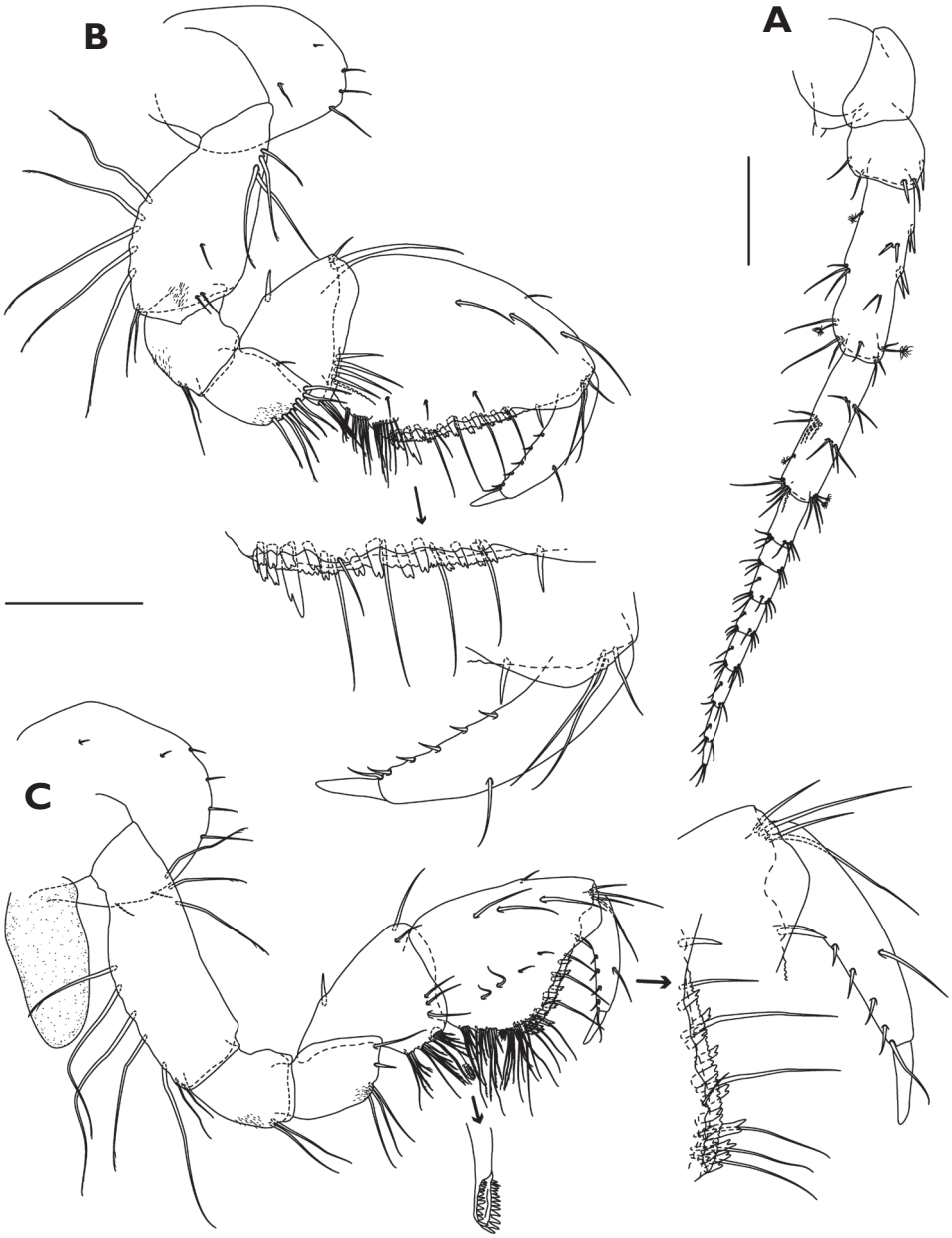

Figure 7. *Stygobromus anacostensis* sp. nov., Allotype female, 5.3 mm (USNM 1606903): **A** antenna 2 **B** gnathopod 1 (palm and dactyl enlarged) **C** gnathopod 2 (rastellate seta, palm and dactyl enlarged). Scale bars: 0.25 mm (**A**); 0.5 mm (**B, C**).

Telson (Fig. 8E). Telson entire, elongated, 1.5× longer than broad, weakly tapering distally; apex with 9 robust setae, 2 plumose setae arise dorsolaterally from both outer margins.

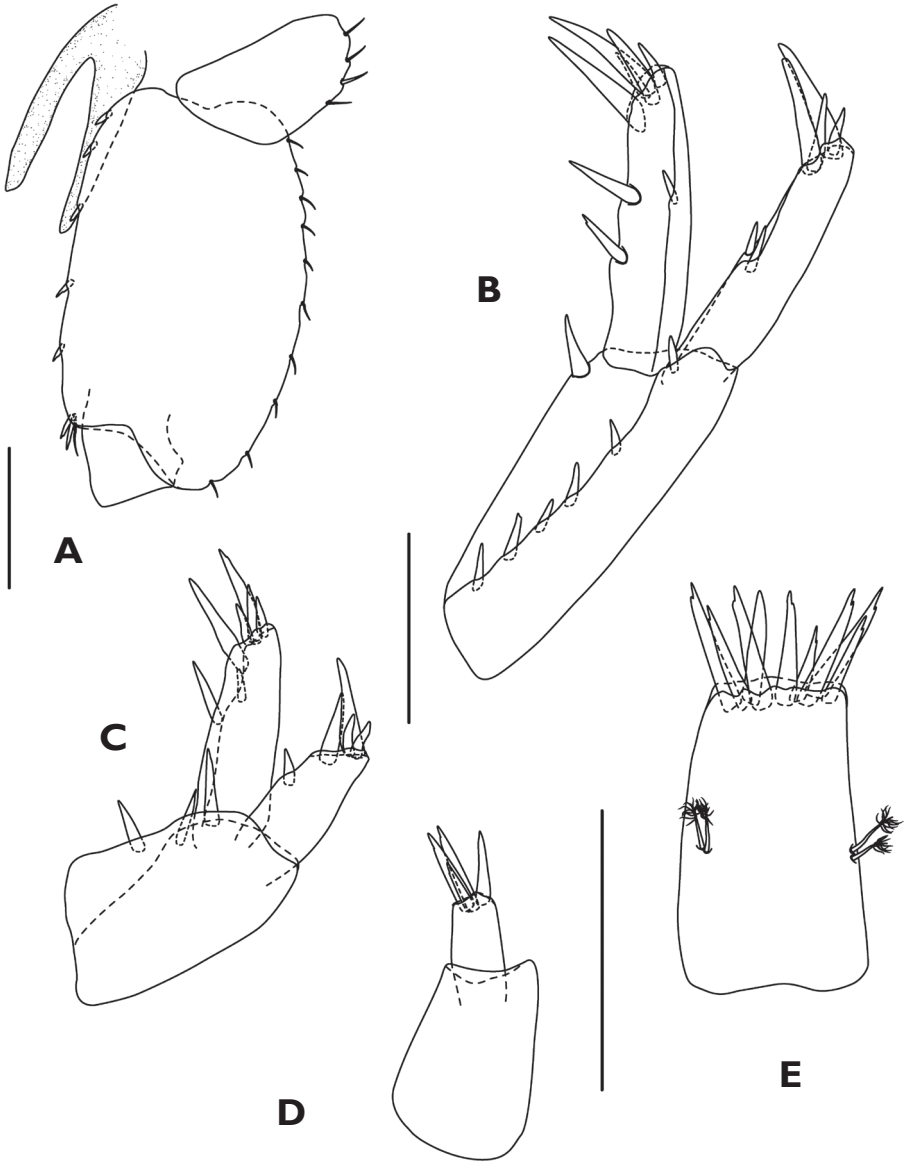

Figure 8. *Stygobromus anacostensis* sp. nov., Allotype female, 5.3 mm (USNM 1606903): **A** coxa and basis of pereopod 7 showing placement of bifurcate sternal gill **B** uropod 1 **C** uropod 2 **D** uropod 3 **E** telson. Scale bars: 0.5 mm (**A–C**); 0.25 mm (**D, E**).

Variation. The new species was shown to vary slightly in several morphological characteristics, particularly between males and females (Table 3).

Molecular diagnosis. Average uncorrected pairwise genetic distance at the mitochondrial *co1* locus between *S. anacostensis* and the most closely related populations of *S. t. potomacus* sampled at Caledon State Park is 6.5%, with 32 fixed mutations separating the two taxa. Between *S. anacostensis* and the closest *S. t. potomacus* population

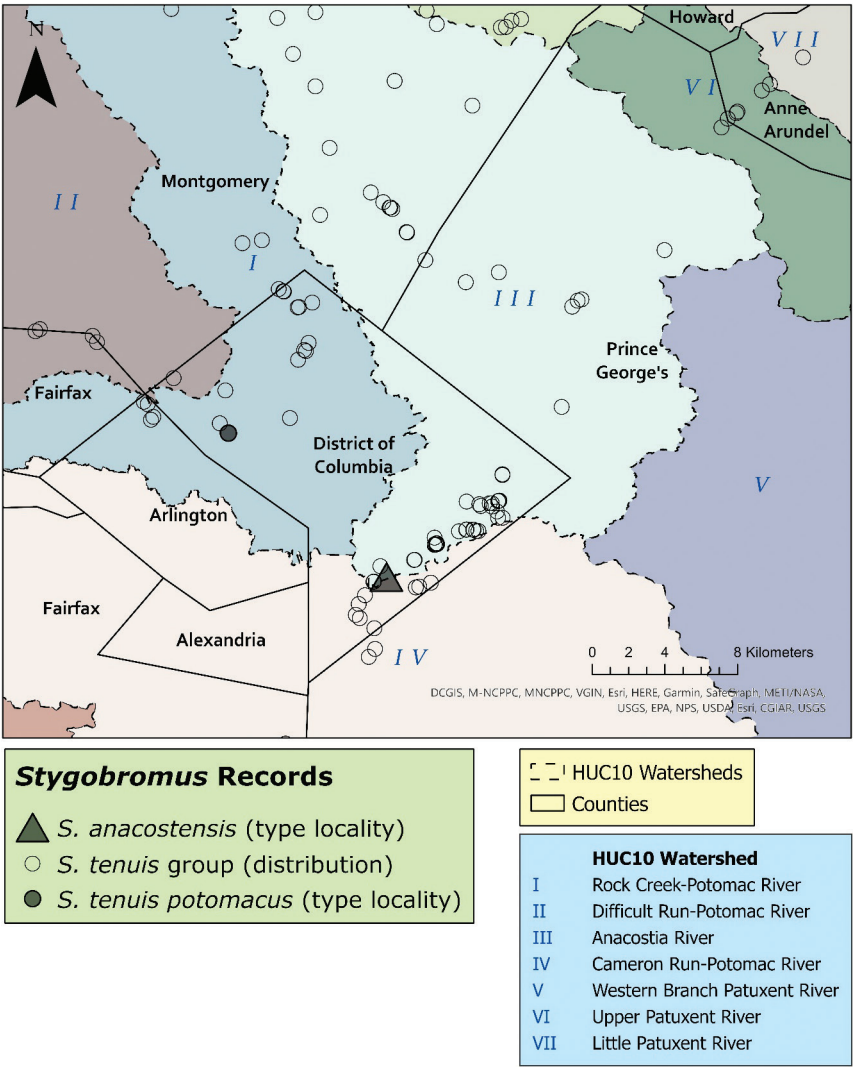

Figure 9. Distribution of *Stygobromus anacostensis* sp. nov. and other *S. tenuis* group species in the Washington D.C. area, USA

(seepage spring near Malcolm X Ave in Anacostia Park; SP101), p-distance is 12.6%, with 67 fixed mutations. P-distance at the mitochondrial *16s* locus between *S. anacostensis* and the populations of *S. t. potomacus* sampled at Caledon State Park is 2.3%, with eight fixed mutations. Nuclear loci exhibited low levels of variation among all *S. tenuis* species group taxa sampled; however, some diagnostic genetic variation was noted. Two fixed mutations in the *h3* locus and one fixed mutation in the *18s* locus exist between *S. anacostensis* and the closest *S. t. potomacus* population (SP101).

Geographical distribution. The species is known to date only from the type locality in Shepherd Parkway, which is a 1200-acre national park located on the southern

Table 3. Variation in morphological characters among select specimens of *Stygobromus anacostensis* sp. nov. examined.

Character	Holotype Male USNM 1606902	Paratype Male USNM 1606904	Allotype Female USNM 1606903	Paratype Female USNM 1606906	Paratype Female USNM 1606905
Body size	5.9 mm	6.7 mm	5.3 mm	4.8 mm	4.8 mm
Antenna 1					
Flagellar segments	18	21	16	16	12
Accessory flagellum	> than 1st flagellar segment	> than 2nd flagellar segment	> than 1st flagellar segment	> than 2nd flagellar segment	sub equal to second flagellar segment
Antenna 2					
Peduncle Segments 4 to 5 length	1.04×	97%	1.15×	1.12×	1.13×
Flagellar segments	unknown	12	7	7	7
Left Mandible					
Palp segment 2 setae	8	7	5	3	4
E-setae	5	5	5	4	3
D-setae	8	8	8	8	5
Right Mantible					
Palp 2nd segment setae	8	5	5	4	4
E-setae	5	5	4	4	3
D-setae	8	8	8	7	5
Maxilla 1					
Inner plate marginal setae	unknown	4	4	3	3
Palp subapical, apical setae	unknown	3, 4	2, 4	2, 4	4, 2
Maxilla 2					
Mx 2 inner plate facial setae	unknown	3	2 or 3	2 or 3	2
Ganthopod 1					
Ischium posterior setae	4	4	2	3	3
Carpus to propdus length	40%	40%	43%	46%	45%
Carpus submarginal setae	6	5	4	4	5
Propodus superior, inferior medial setae	4, 6	2, 4	3, 3	3, 3	2, 4
Palm inner, outer bifid setae	7, 7	7, 6	5, 5	4, 4	5, 3
Ganthopod 2					
Coxal plate apical, facial setae	4,1	4,1	6,2	4,1	3,0
Ischium posterior setae	3	4	2	2	3
Merus anterior margin robust seta	1	1	0	0	0
Carpus to propdus length	71%	64%	82%	79%	84%
Carpus rastellate setae	3	3	1	3	0
Propodus superior, inferior medial setae	5, 5	3, 4	3, 4	3, 3	3, 3
Palm inner, outer bifid setae	5, 5	5, 6	3, 3	3, 3	3, 4
Dactylus inner setae	1	1	5	4	1
Pereopod 5					
Coxal plate anterior apical setae	0	0	2	1 to 2	2
Basis posterior serrations	9	11	8	8	8
Pereopod 7					
Coxal plate posterior apical setae	4	3	4	3	2
Basis anterior setae	8	7	5	5	4
Epimera					
Epimeron 2 ventral, posterior setae	3, 2	unknown	3, 2	2, 2	2, 4

Character	Holotype Male USNM 1606902	Paratype Male USNM 1606904	Allotype Female USNM 1606903	Paratype Female USNM 1606906	Paratype Female USNM 1606905
Uropods					
Uropod 1 peduncle outer, inner setae	8, 1	10, 1	6, 1	9, 1	8, 1
Uropod 2 peduncle outer, inner setae	2, 2	3, 1	2, 1	2, 1	2, 1
Uropod 2 outer ramus outer, inner setae	0, 0	1, 0	1, 0	1, 0	0, 0
Uropod 2 outer ramus apical setae	4	3	4	4	5
Uropod 2 inner ramus outer, inner setae	2, 2	2, 1	1, 1	1, 1	1, 1
Uropod 3 ramus setae	3	4	4	3	2
Telson apical robust setae	10	10	9	9	8

Figure 10. The type locality of *S. anacostensis* is a small hypotelminorheic seepage spring just off of Malcolm X Avenue, Shepherd Parkway, Washington, D.C., USA. Photograph by Jenna Keany.

bank of the Anacostia River just upstream from where the river flows into the Potomac River (Fig. 9). Shepherd Parkway is part of National Capital Parks East (NACE).

Habitat and ecology. Like other species of *Stygobromus*, *S. anacostensis* is a stygobiotic species occurring in groundwater habitats. All specimens have been collected from a seepage spring just off Malcolm X Avenue SE that marks the resurgence of hypotelminorheic groundwater at the surface (Fig. 10). Amphipods have been observed and collected from underneath moss-covered rocks, moss, and leaf litter on the small, 2-m high rockface as well as the small pool of the seepage spring. The seepage spring possesses water throughout most of the year Little is known regarding the ecology and life history currently. *Stygobromus anacostensis* co-occurs with the groundwater isopod *Conasellus* (=*Caecidotea*) *kenki* (Bowman, 1967).

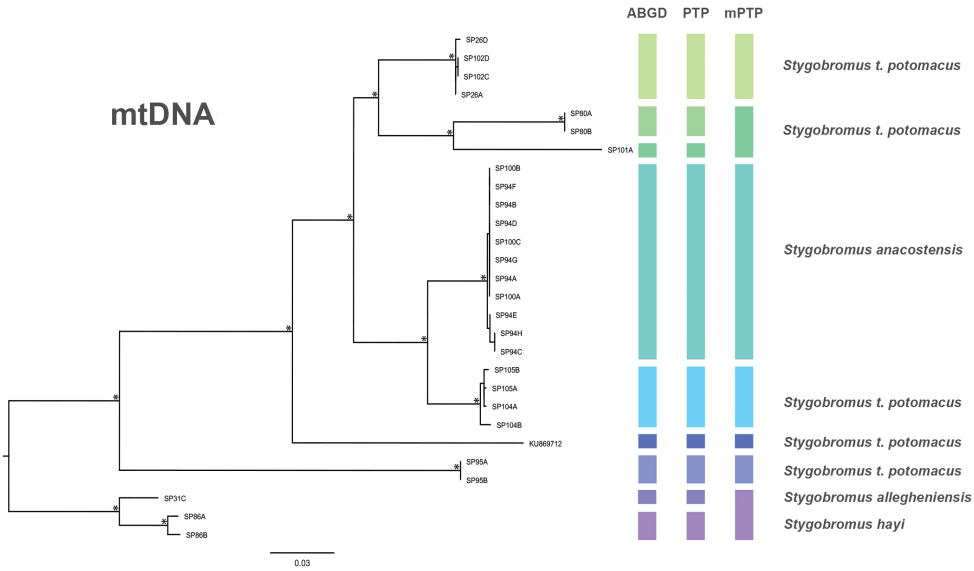

Figure 11. Maximum-likelihood phylogeny and species delimitations of *Stygobromus anacostensis* and other *S. tenuis* species group taxa for the mtDNA dataset (*co1+16s* loci). Asterisk represents bootstrap node support greater than 90. Colored bars represented hypothesized MOTU groupings (i.e., species) based on corresponding delimitation analyses.

Conservation. *Stygobromus anacostensis* is known only from the type locality. The NatureServe conservation rank calculated is Critically Imperiled (G1). Under IUCN Red List criteria, *S. anacostensis* was assessed as Critically Endangered (CR B1) because of an extremely small EOO and AOO (known from a single small seep) in an urban area. Major threats to the species include increased risk of human intrusion and disturbance, habitat degradation, and pollution. The type locality population is offered some protection by occurring on National Park Service land, but the area controlled by the NPS is very narrow, and the site is highly vulnerable to road salt as well as any attempt to "improve" the drainage in the vicinity of the sidewalk.

Genetic and phylogenetic analyses

We amplified in total 2,738 bp of five loci. Uncorrected mtDNA p-distance between *S. anacostensis* and populations of *S. tenuis potomacus* at Caledon State Park (SP104 and SP105) was 6.5% and 12.6% between *S. anacostensis* and the nearest *S. t. potomacus* population sampled in Anacostia Park (SP101). Average uncorrected nucDNA p-distance was substantially lower, averaging 0.001 between *S. anacostensis* and *S. tenuis potomacus* at Caledon State Park (SP104 and SP105), and 0.004 between *S. anacostensis* and the nearest *S. t. potomacus* population sampled in Anacostia Park (SP101).

The optimal substitution models for first, second, and third positions of *co1* were TrNef+I (Tamura and Nei 1993), F81 (Felsenstein 1981), and K81+I (Kimura 1981), respectively. The optimal substitution model was HKY + I + G (Hasegawa et al. 1985)

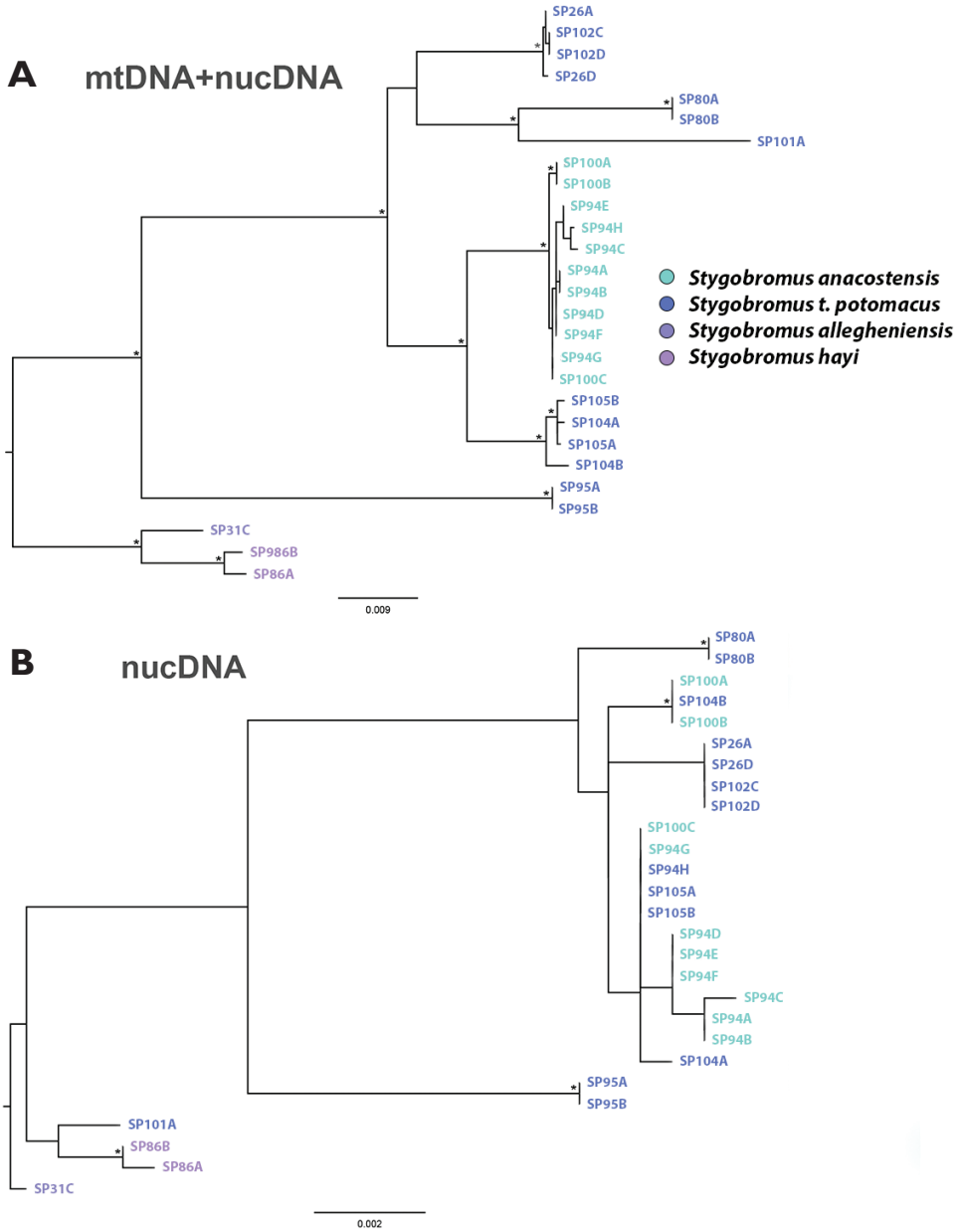

Figure 12. Maximum-likelihood phylogenies of of *Stygobromus anacostensis* and other *S. tenuis* species group taxa for the (**A**) mtDNA+nucDNA dataset (*co1+16s+18s+28s+h3* loci) and (**B**) nucDNA dataset (*18s+28s+h3* loci). Asterisk represents bootstrap node support greater than 90.

for *16s*, K80 (Kimura 1980) for *18s*, TIM2+I for *28s*, and JC (Jukes and Cantor 1980) for *h3*. Phylogenetic tree topologies obtained for ML and Bayesian inference were highly similar. Phylogenetic trees reconstructed using both ML and Bayesian inference for the mtDNA (*co1+16s*; Fig. 11) and mtDNA+nucDNA datasets (Fig. 12A) delimited

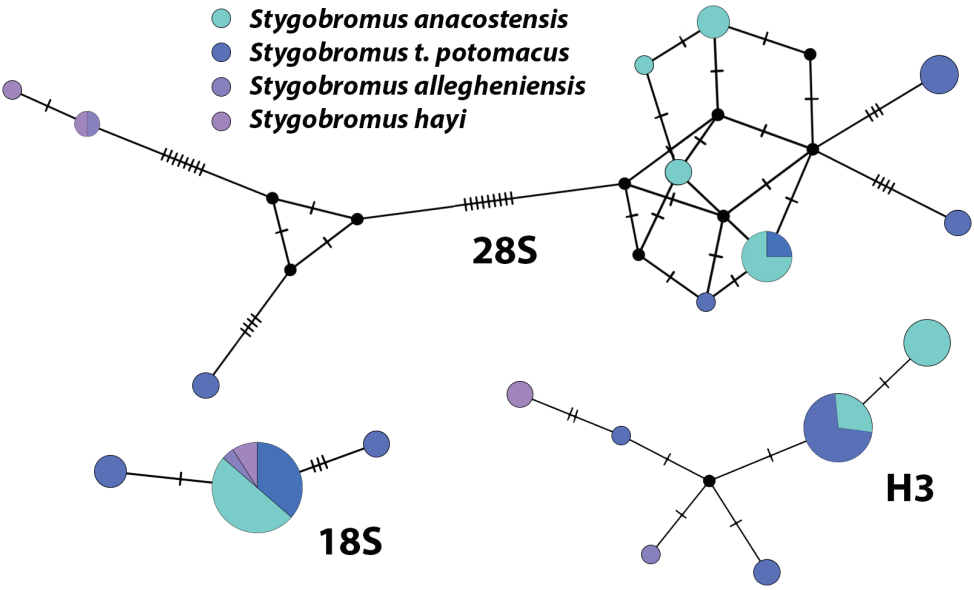

Figure 13. Median joining networks for nuclear loci (*18s*, *28s*, and *h3*) generated in PopART v1.7.

individuals of *Stygobromus anacostensis* from the type locality as distinct from other populations of the *S. tenuis* species group sampled with high bootstrap support. Populations of *S. tenuis potomacus* did not form a monophyletic group for any dataset. Nuclear loci exhibited low levels of variation among all *S. tenuis* species group taxa sampled (Figs 12B, 13). Two fixed mutations in the *h3* locus and one fixed mutation in the *18s* locus exist between *S. anacostensis* and the closest *S. t. potomacus* population (SP101).

Species delimitation

For the mtDNA dataset (Fig. 11), the ABGD approach resulted in nine MOTUs, with convergence of initial and recursive partitions at prior intraspecific divergence (P) = 0.028, which remained stable until P = 0.0359. The PTP approach yielded the same MOTU delimitations. All *S. anacostensis* samples formed a MOTU, while several *S. tenuis potomacus* populations were delimited as distinct MOTUs. The mPTP approach estimated seven MOTUs, with highly similar designations to the ABGD delimitations. *Stygobromus anacostensis* individuals were grouped as a single MOTU, as were several *S. tenuis potomacus* populations. *Stygobromus allegheniensis* and *S. hayi* were grouped into a single MOTU.

Discussion

Stygobromus anacostensis is morphologically and genetically most similar to *S. tenuis potomacus*, which overlaps in distribution with the new species. However, several morphological characters readily distinguish the two species in the Washington D.C.

area, including by having only 2 C-setae on the mandibular palp and up to 8 E-setae. *Stygobromus anacostensis* also shares a similar overall morphology with other members of the *S. tenuis* species group as defined by Holsinger (1978) in the region, but the new species can be distinguished morphologically from other members of the species complex by possessing a serrate blade-like edge along the length of both palms of gnathopods 1 and 2 and by possessing rastellate setae on the posterodistal margin of the carpus of gnathopod 2. It should be noted that the serrate blade-like edge along the length of both gnathopod palms was most discernable after the digestion protocol noted in the materials and methods. However, this feature was also easily visible on nondigested specimens, including juveniles. Thus, the characteristic is not an artifact of the digestion protocol. It is possible that this feature may be found on other *Stygobromus* species but has never been documented prior to this analysis. If so, this characteristic may be diagnostic not by its presence, but by its degree, as it was so highly visible. A reexamination of the palms of other *Stygobromus* species will help to determine the status of this characteristic.

With the description of *S. anacostensis*, the total number of described stygobiotic amphipods from the Piedmont and Atlantic Coastal Plain of the Maryland, Virginia, and District of Columbia area is now 14 species, with eight species now known from hypotelminorheic habitats in and near the Washington D.C. metropolitan area. Interestingly, unlike many other seepage springs in the region (Culver et al. 2012), *S. anacostensis* is not known to co-occur with any other *Stygobromus* species. *Stygobromus tenuis potomacus* is known from a seepage spring one km from the type locality in Anacostia Park, although in a different HUC10 drainage, but the possibility exists that this species co-occurs with *S. anacostensis*.

The discovery of a new species of *Stygobromus* amphipod from the Piedmont and Atlantic Coastal Plain is not surprising given high species richness of the genus not only in the region but also throughout North America, and the description of several species in recent years throughout the United States (Holsinger et al. 2011; Holsinger and Ansell 2014; Holsinger and Sawicki 2016; Cannizzaro et al. 2019; Gibson et al. 2021). Moreover, uncovering cryptic diversity is an increasingly common finding of population genetic and phylogenetic studies in groundwater fauna (Lefébure et al. 2006; Murphy et al. 2009; Zakšek et al. 2009; Niemiller et al. 2012, 2013; Devitt et al. 2019), including crangonyctid amphipods (Etheridge et al. 2013; Niemiller et al. 2018; Cannizzaro et al. 2020). Niemiller et al. (2018) uncovered cryptic genetic variation at the mitochondrial *co1* locus among populations of *S. tenuis potomacus* sampled in the Washington D.C. area, including up to 9.2% sequence divergence among populations separated by only 7.2 km straight-line distance. We uncovered similar levels of genetic variation among and within species of the *S. tenuis* species group highlighted by 12.6% mtDNA sequence divergence between populations of *S. anacostensis* and *S. t. potomacus* separated by just 10 km. Such levels of divergence support the view that many groundwater species are dispersal limited and that morphological species with broader distributions are likely comprised of multiple morphologically similar but genetically distinct lineages (Niemiller et al. 2012, 2018; Ethridge et al. 2013). *Stygobromus anacostensis* is one of likely several additional undescribed species that await morphological and genetic investigation and formal description within the *S. tenuis* species group.

Acknowledgments

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Why cave planthoppers study matters: are Cixiidae a subtroglophile lineage? (Hemiptera, Fulgoromorpha)

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Abstract

Planthoppers are an interesting and contrasting model among insects for studying the subterranean environments. Their morphological and ethological adaptations to the underground conditions (complete darkness, lower temperatures, high hygrometry, stability of environmental constants, rarefied food sources, etc.), and their worldwide distribution in both temperate and tropical areas make them an interesting model among invertebrates. In this review, we highlight why cave planthoppers study matters, with particular emphasis on the Cixiidae. The two hypotheses proposed, the 'climatic relict hypothesis' and the 'adaptive shift', are not sufficient enough to clearly understand and explain the drivers to cavernicoly. Phylogenetic analyses approaches might help to better document and increase our knowledge on such peculiar environments. The singularity of the distribution pattern of the adaptation to cavernicoly in planthoppers raises also interesting questions to investigate and suggest contrasting scenarios to explore further, particularly should the Cixiidae be defined as a subtroglophile lineage?

Keywords

Cave, cavernicoly, hypogean, Kinnaridae, Meenoplidae, subterranean

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Introduction

When Austro-Hungarian entomologist Ferdinand Schmidt in 1832 described the first beetle species adapted to caves in Postojna Cave, Slovenia (Schmidt 1832; Polak 2005), he also revealed the existence of subterranean insect life stable and suitable. Until then unsuspected, this ability to adapt to underground life is now rich in many examples and one now recognizes a real "hidden" underground diversity where all the major phyla are now represented (Sendi et al. 2020). Indeed, since then, interest in subterranean habitats has continued to grow (Mammola 2019), with all authors emphasizing the great potential of their study and how the subterranean environment is a well-suited model for studying the processes of adaptation of organisms under various morphological, ethological or ecological perspectives (Racovitza 1907; Jeannel 1926; Vandel 1964; Poulson and Culver 1969; Howarth 1980; Gibert and Deharveng 2002). While substantial results have already been published, the subterranean world keeps fascinating and questioning scientists, who consider it a natural laboratory of well-suited models for evolutionary and ecological studies (Poulson and White 1969; Culver and Pipan 2010; Ribera et al. 2018).

Focusing on the insect fauna only, hypogean species occur in 19 of the insect orders (Romero 2009). In Hemiptera, even if the obligate phytophagous Auchenorrhyncha Fulgoromorpha would not be the first expectation in the subterranean environment (Hoch 2002), more than 60 species of planthoppers have now been described and documented as subterranean species (Bourgoin 2024). This may seem low compared to Coleoptera where the number and diversity of species are the greatest, thus concentrating the majority of the studies (Gibert and Deharveng 2002; Faille et al. 2015a; Huang 2022). However, the obligatory phytophagy constraints of planthoppers, their short-range intraspecific communication transmitted by the substrate (Claridge and Vrijer 1994), which direct their reproductive behavior, their morphological and ethological adaptations to the underground conditions (complete darkness, lower temperatures, high hygrometry, stability of environmental constants, rarefied food sources, etc.), and their worldwide distribution in both temperate and tropical areas, make planthoppers an interesting and contrasting model among insects for studying the subterranean environments.

Based on these singularities, the purpose of this review is to summarize our current knowledge on cave planthoppers, with particular emphasis on the Cixiidae. We point to possible future research perspectives by using these taxa as models to further explore the mechanisms of adaptation to a highly restrictive environment, and by documenting the resulting phylogenetic patterns we observe (Barr 1968; Protas and Jeffery 2012; Howarth and Moldovan 2018a; Soares and Niemiller 2020; Huang 2022).

Materials and methods

When examining subterranean ecosystems, and in contrast to the surface-dwelling species inhabiting epigean habitats, two primary categories of inhabitants are distinguished: soil-dwelling species residing in endogeic habitats, and cave-dwelling species residing in hypogean habitats. Among the cave-dwelling species, numerous authors have attempted to

categorize them based on various criteria such as morphological, physiological, ethological, or ecological (summarized in Howarth and Moldovan 2018b). However, adaptability to underground environments exists along a continuum. Consequently, regardless of the chosen classification criterion, whether it be, subjectivity often prevails. In this review, we will follow Sket's 2008 classification, rooted in the "Schiner-Racovitza classification" (Trogloxene / Troglophile / Troglobiont), which is considered the foundation for all subsequent categorizations (Horvath and Moldovan 2018b), with the following definitions:

- Troglobiont species strongly linked to underground ecosystems.
- Eutroglophile species, epigean but able to maintain a permanent subterranean population.
- Subtroglophile species perpetually or temporarily inhabiting a subterranean habitat but linked to the epigean habitats for some biological functions.
- Trogloxene species occurring sporadically in a hypogean habitat and unable to maintain a subterranean population.

The map was built using the software QGIS 3.10.2 and we used the climate zones proposed by van Velthuisen et al. in 2007.

What do we know about cave planthoppers?

Geographic distribution

The first cave-dwelling planthopper was mentioned in 1907 by the Rumanian biologist Emil Racovitza who reported the observation of an unpigmented cixiid planthopper which he identified as "*Cixius* sp." from the Balearic Island of Mallorca (Racovitza 1907). Unfortunately, the species was not described formally, and there is no record of any voucher specimens. Only nearly half a century later, another subterranean planthopper species was found in Zimbabwe. The species displays distinct modifications from epigean species, such as the lack of ocelli and obsolete compound eyes, vestigial, pad-like tegmina and light body pigmentation. It was reported as "a subterranean maggot-like planthopper" (China and Fennah 1952: 189), living in the soil, apparently feeding on roots of maize and tobacco, and being tended by ants. The species was so much modified, that it could not be accommodated in any of the existing Fulgoromorpha families, it was described in a new family, Hypochthonellidae China & Fennah, 1952, for *Hypochthonella caeca* China & Fennah, 1952. The genus to date remains monospecific.

Since then, cavernicolous planthopper species have been discovered from many parts of the world (Fig. 1): Argentina, Australia and New Zealand, Brazil, Canary Islands, Hawaii, Mexico, Madagascar and La Réunion and several countries in Europe (Croatia, France, Italy, Slovenia, Spain, France) (Fig. 1). To date, 70 planthopper species in five planthoppers families have been explicitly reported to live in the subterranean ecosystems (Table 1): Cixiidae Spinola, 1839 (44 species), Delphacidae Leach, 1815 (3 species), Meenoplidae Fieber, 1872 (14 species), Kinnaridae Muir, 1925 (7 species), Hypochthonellidae (1 species) and

Table 1. The cave-dwelling species.

Family	Species	Authorship	Subterranean habitat	Type locality	Ecological category
Cixiidae	*Borysthenes hainanensis*	Lyu & Webb, 2023	Lava tube and epygean	Quishierdong lava tube, Haikou, Hainan, China	Eutroglophile
Cixiidae	*Brixia briali*	Hoch & Bonfils, 2003	Lava tube	Caverne de la tortue, La Réunion	Troglobiont
Cixiidae	*Celebenna thomarosa*	Hoch & Wessel, 2011	Limestone cave	Gua Assuloang, Maros karst, Sulawesi, Indonesia	Troglobiont
Cixiidae	*Cixius actunus*	Hoch, 1988	Limestone cave	Cueva de las Maravillas, Oaxaca, Mexico	Troglobiont
Cixiidae	*Cixius ariadne*	Hoch & Ashe, 1993	Lava tube	Cueva de la Curva, El Hierro, Canary Islands	Troglobiont
Cixiidae	*Cixius azopicavus*	Hoch, 1991	Lava tube	Furna de Agostinha, Pico, Azores	Troglobiont
Cixiidae	*Cixius cavazoricus*	Hoch, 1991	Lava tube	Furna dos Concheiros, Faial, Azores	Troglobiont
Cixiidae	*Cixius nycticolus*	Hoch & Ashe, 1993	Lava tube	Cueva Roja, El Hierro, Canary Islands	Troglobiont
Cixiidae	*Cixius orcus*	Fennah, 1973	Limestone cave	Cueva de Emilia, Queretaro, Mexico	Troglobiont
Cixiidae	*Cixius palmeros*	Hoch & Ashe, 1993	Lava tube	Cueva de los Palmeros, La Palma, Canary Islands	Troglobiont
Cixiidae	*Cixius pinarcoladus*	Hoch & Ashe, 1993	Lava tube	Cueva del Diablo, La Palma, Canary Islands	Troglobiont
Cixiidae	*Cixius ratonicus*	Hoch & Ashe, 1993	Lava tube	Cueva del Raton, La Palma, Canary Islands	Troglobiont
Cixiidae	*Cixius tacandus*	Hoch & Ashe, 1993	Lava tube	Cueva de Tacande, La Palma, Canary Islands	Troglobiont
Cixiidae	*Coframalaxius bletteryi*	Le Cesne & Bourgoin, 2022	Limestone cave	Grotte de la Chèvre d'Or, Alpes-Maritimes, France	Troglobiont
Cixiidae	*Confuga persephone*	Fennah, 1975	Limestone cave	Council cave, Takaka, Nelson province, New Zealand	Troglobiont
Cixiidae	*Ferricixius davidi*	Hoch & Ferreira, 2012	Ferrugenous cave	MP-08 cave, Itabirito, Minas Gerais state, Brazil	Troglobiont
Cixiidae	*Ferricixius goliathi*	Santos, Hoch & Ferreira, 2023	Ferrugenous cave	ABOB-0043 cave, Nova Lima, Minas Gerais state, Brazil	Troglobiont
Cixiidae	*Ferricixius michaeli*	Santos, Hoch & Ferreira, 2023	Limestone cave	ICMAT-0053 cave, Matozinhos, Minas Gerais state, Brazil	Troglobiont
Cixiidae	*Ferricixius urieli*	Santos, Hoch & Ferreira, 2023	Quartz	Casas cave, Lima Duarte, Minas Gerais state, Brazil	Subtroglophile
Cixiidae	*Ibleocixius dunae*	D'urso & Grasso, 2009	Limestone cave	Iblei mountains, Sicily	Troglobiont
Cixiidae	*Iolania frankanstonei*	Hoch & Porter, 2024	Lava tube	Kipuka Kanohina system, Hawaii	Troglobiont
Cixiidae	*Notolathrus sensitiva*	Remes-Linecov, 1992	Limestone cave	Caverna del Arenal, sistema de Cuchillo Cura, Neuquen, Argentina	Troglobiont
Cixiidae	*Oliarus gagnei*	Hoch & Howarth, 1999	Lava tube	Ulupalakua cave, Maui Island, Hawaii	Troglobiont
Cixiidae	*Oliarus hernandezi*	Hoch & Izquierdo, 1996	Lava tube	Finch cave, Floreana Island, Galapagos	Troglobiont
Cixiidae	*Oliarus kalaupapae*	Hoch & Howarth, 1999	Lava tube	Fisherman Shak's cave #1, Molokai Island, Hawaii	Troglobiont
Cixiidae	*Oliarus lorettae*	Hoch & Howarth, 1999	Lava tube	Ana Lima Kipo lava tube, Kiholo bay, Hawaii	Troglobiont
Cixiidae	*Oliarus makaiki*	Hoch & Howarth, 1999	Lava tube	Yellow Jacket cave, Hualalai volcano, Hawaii	Troglobiont
Cixiidae	*Oliarus polyphemus*	Fennah, 1973	Lava tube	Bird Park cave, Kipuka Puaulu, Hawaii	Troglobiont
Cixiidae	*Oliarus priola*	Fennah, 1973	Lava tube	Holoinawawai stream cave, Maui Island, Hawaii	Troglobiont
Cixiidae	*Oliarus waikau*	Hoch & Howarth, 1999	Lava tube	Waikau cave, Maui Island, Hawaii	Troglobiont
Cixiidae	*Sanghabenna florenciana*	Hoch & Bourgoin, 2017	chaos of granite blocks	Hon Ba massif, Vietnam	Subtroglophile
Cixiidae	*Solonaima baylissa*	Hoch & Howarth, 1989	Lava tube	Bayliss cave, Mt Surprise, Queensland, Australia	Troglobiont

Family	Species	Authorship	Subterranean habitat	Type locality	Ecological category
Cixiidae	*Solonaima halos*	Hoch & Howarth, 1989	Limestone cave	Queenslander cave, Chillagoe, Queensland, Australia	Troglobiont
Cixiidae	*Solonaima irvini*	Hoch & Howarth, 1989	Limestone cave	Swiftlet scallops cave, Chillagoe, Queensland, Australia	Troglobiont
Cixiidae	*Solonaima pholetor*	Hoch & Howarth, 1989	Limestone cave	Royal Arch cave, Chillagoe, Queensland, Australia	Troglobiont
Cixiidae	*Solonaima stonei*	Hoch & Howarth, 1989	Limestone cave	Arena cave, Chillagoe, Queensland, Australia	Troglobiont
Cixiidae	*Solonaima sullivani*	Hoch & Howarth, 1989	Limestone cave	Crystal cascades cave, Mt Mulgrave station, Queensland, Australia	Troglobiont
Cixiidae	*Tachycixius crypticus*	Hoch & Ashe, 1993	?	Palo blanco, Tenerife, Canary Islands	Troglobiont
Cixiidae	*Tachycixius lavatubus*	Remane & Hoch, 1988	Lava tube	Cueva Grande de Chio, Tenerife, Canary Islands	Troglobiont
Cixiidae	*Tachycixius retrusus*	Hoch & Ashe, 1993	?	Barranco de Ijuana, Tenerife, Canary Islands	Troglobiont
Cixiidae	*Trigonocranus emmeae*	Fieber, 1876	Endogean and epygean	Emme valley, Switzerland	Eutroglophile
Cixiidae	*Trirhacus helenae*	Hoch, 2013	Dolomite cave	Spilja kod Nerezinog dola, Mljet Island, Croatia	Troglobiont
Cixiidae	*Typhlobrixia namorokensis*	Synave, 1953	Limestone cave	Namoroka karst, Madagascar	Troglobiont
Cixiidae	*Undarana collina*	Hoch & Howarth, 1989	Lava tube	Collins 210 cave, Mt Surprise, Queensland, Australia	Troglobiont
Cixiidae	*Undarana rosella*	Hoch & Howarth, 1989	Lava tube	Bayliss cave, Mt Surprise, Queensland, Australia	Troglobiont
Delphacidae	*Notuchus kaori*	Hoh & Ashe, 2006	Endogean	Pic du grand Kaori, New Caledonia	Troglobiont
Delphacidae	*Notuchus larvalis*	Fennah, 1980	Limestone cave	Taphozous cave, Hienghène, New Caledonia	Troglobiont
Delphacidae	*Notuchus ninguae*	Hoch & Ashe, 2006	Endogean	Pic Ningua, New Caledonia	Troglobiont
Flatidae	*Budginmaya eulae*	Fletcher, 2009	Endogean	Nid de *Camponotus*, Bandalup Hill, Western Australia	Troglobiont
Hypochthonelidae	*Hypochthonella caeca*	China & Fennah, 1952	Endogean	Salisbury, Southern Zimbabwe	Troglobiont
Kinnaridae	*Iuiuia caeca*	Hoch & Ferreira, 2016	Limestone cave	Lapa de Baixão cave, Bahia, Brazil	Troglobiont
Kinnaridae	*Oeclidius antricola*	Fennah, 1980	Limestone cave	Jackson Bay cave, Clarendon, Jamaica	Troglobiont
Kinnaridae	*Oeclidius hades*	Fennah, 1973	Limestone cave ?	Cueva de Valdosa, San Luis Potosi, Mexico	Troglobiont
Kinnaridae	*Oeclidius minos*	Fennah, 1980	Limestone cave	Jackson Bay cave, Clarendon, Jamaica	Troglobiont
Kinnaridae	*Oeclidius persephone*	Fennah, 1980	Limestone cave	Portland caves, Clarendon, Jamaica	?
Kinnaridae	*Kinnapotiguara troglobia*	(Hoch & Ferreira, 2013)	Limestone cave	Gruta do troglobio, Rio Grande do Norte, Brazil	Troglobiont
Kinnaridae	*Valenciolenda fadaforesta*	Hoch & Senda, 2021	Dolomitic cave	Valencia, Vilamarxant, 'Murceliagos' cave, Spain	Troglobiont
Meenoplidae	*Eponisia hypogaea*	Hoch, 1996	Limestone cave	Grottes d'Adio, New Caledonia	Troglobiont
Meenoplidae	*Meenoplus cancavus*	Remane & Hoch, 1988	Lava tube	Cueva Don Justo, El Hierro, Canary Islands	Troglobiont
Meenoplidae	*Meenoplus charon*	Hoch & Ashe, 1993	Lava tube	Cueva de la Curva, El Hierro, Canary Islands	Troglobiont
Meenoplidae	*Meenoplus claustrophilus*	Hoch & Ashe, 1993	Lava tube	Cueva del Raton, La Palma, Canary Islands	Troglobiont
Meenoplidae	*Meenoplus roddenberryi*	Hoch & Naranjo, 2012	Lava tube	Minas los Roques, Gran Canaria, Canary Islands	Troglobiont
Meenoplidae	*Nisia subfogo*	Hoch & Oromi, 1999	Lava tube	Caldera de Fogo, Fogo, Cape Verde Islands	Troglobiont

Family	Species	Authorship	Subterranean habitat	Type locality	Ecological category
Meenoplidae	*Phaconeura capricornia*	Hoch, 1990	Limestone cave	Swiss cheese cave, Cape York, Queensland, Australia	Troglobiont
Meenoplidae	*Phaconeura crevicola*	Hoch, 1990	Limestone cave	Raindance cave, Queensland, Chillagoe, Australia	Troglobiont
Meenoplidae	*Phaconeura minyamea*	Hoch, 1990	Limestone cave	Tea tree cave, Queensland, Chillagoe, Australia	Troglobiont
Meenoplidae	*Phaconeura mopamea*	Hoch, 1990	Limestone cave	Carpentaria cave, Queensland, Chillagoe, Australia	Troglobiont
Meenoplidae	*Phaconeura pluto*	Fennah, 1973	Limestone cave	Quandong cave, Nambung national park, Western Australia	Troglobiont
Meenoplidae	*Phaconeura proserpina*	Hoch, 1993	Limestone cave	Cave C-215, North west cape peninsula, Western Australia	Troglobiont
Meenoplidae	*Suva oloimoa*	Hoch & Ashe, 1988	Lava tube	Oloimoa cave, Savai'i Island, Samoa	Troglobiont
Meenoplidae	*Tsingya clarkei*	Hoch & Wessel, 2014	Limestone cave	Anjohy Manitsy, Tsingy de Bemaraha, Madagascar	Troglobiont

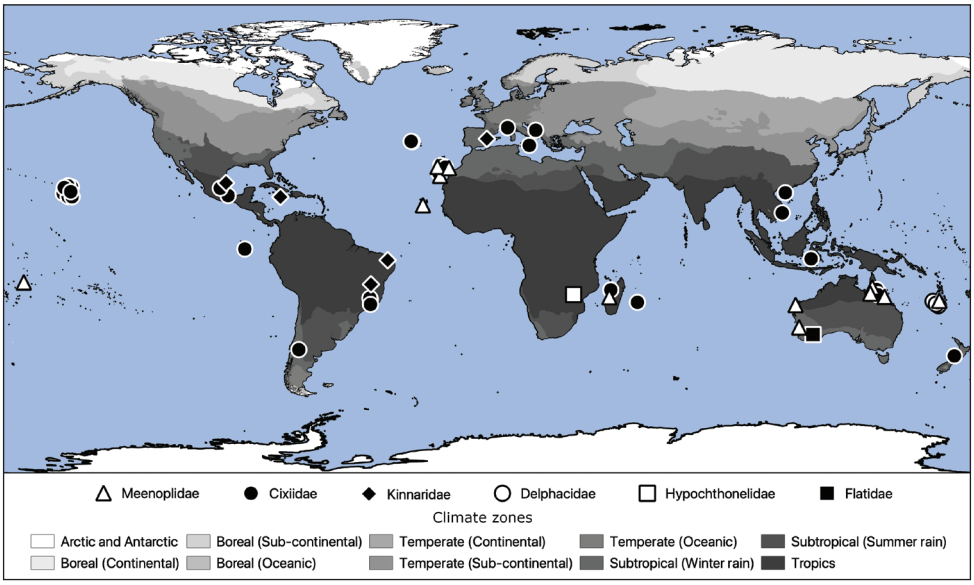

Figure 1. Distribution map of the cave-dwelling planthoppers according to main world ecoregions.

Flatidae (1 species) (Hoch 1994; Hoch 2013, and references therein; Bourgoin 2024). A species of Flatidae from Australia, discovered under conditions similar to those of *H. caeca* – within an ant nest beneath a rock, exhibiting "morphological adaptations akin to those observed in cave-dwelling planthoppers" (Fletcher and Moir 2009) – is also included in this list. Most of these species (58 species) are true troglobionts exhibiting troglomorphies being adaptations correlated with cavernicoly.

Obviously, it is very likely that many new species remain to be discovered as numerous vast known cave systems all around the world are still to be explored (Hoch 2002). Only in the past two decades many new discoveries were reported from Papua New Guinea (Hoch 2002), Brazil (Hoch and Ferreira 2012, 2016; Souza Silva et al. 2020; Santos et al. 2023), Madagascar (Hoch et al. 2014), Vietnam (Hoch et al. 2017),

and even in better explored areas in Europe such as in Italy (D'Urso and Grasso 2009), Canary Islands (Hoch et al. 2012), Croatia (Hoch 2013), Spain (Hoch et al. 2021), or France (Le Cesne et al. 2022).

Phylogenetic patterns of cavernicoly in planthoppers

With few exceptions, only two main lineages within the Fulgoromorpha, the Cixiidae and the Meenoplidae-Kinnaridae have succeeded in colonizing underground ecosystems. These belong to two different superfamilies (Delphacoidea and Fulgoroidea respectively) (Bourgoin and Szwedo 2023) and are therefore phylogenetically independent (Bucher et al. 2023). Both are regarded as groups of epigean species, with larval instars feeding on roots known to be living close to or inside the soil (Hoch 1994, 2002; Wessel et al. 2007; Bowser 2014; Bartlett et al. 2018). Particularly in Cixiidae, all basal lineages and several tribes (Luo et al. 2021; Bourgoin et al. 2023a) are represented: Bennini Metcalf, 1938 (2 species), Brixiini Emeljanov, 2002 (9 species), Cixiini Spinola, 1839 (16 species), Oecleini Muir, 1922 (4 species), Pentastirini Emeljanov, 1971 (8 species), Pintaliini Metcalf, 1938 (1 species).

Aside from these three families, three cavernicolous Delphacid species, all belonging to the same genus *Notuchus* Fennah, 1969 from New Caledonia should also be mentioned. Interestingly and as for several cixiid species also (Bourgoin et al. 2023b), at least two of them are being tended by ants (see Hoch et al. 2006) such as the Hypochthonellid species. The latter displays so many troglomorphic characters (depigmented, micropterism, blindness, maggot-like habitus) that until now, the family remains unplaced and might be related to Flatidae (Bartlett et al. 2018). Another Western Australian flatid species, *Budginmaya eulae* Fletcher & Moir, 2009, also tended by ants, exhibits reduction of the tegmina, hindwings and eyes, pale coloration and increased number of setae on the head, body, tegmina and legs (Fletcher and Moir 2009).

Cavernicolous planthopper biology

Environment

The subterranean biome ranges from the 'Milieu Souterrain Superficiel' (MSS, Juberthie et al. 1980), a 'network of empty air-filled voids and tiny cracks developed between rock fragments' under the topsoil (also often referred as the Mesovoid Shallow Substratum, Mammola et al. 2016), to narrow dark rifts and crevices, more or less deep wells, connected or not to the surface (extended transition zone), to small entirely lightless, interconnected voids and cavities of the 'Milieu Souterrain Profond' (MSP, Juberthie 1983) to caves. These can reach large dimensions and extensions, up to several meters high and many kilometers of passage (e.g., Allred and Allred 1997). Subterranean planthoppers have been found in this wide variety of subterranean ecosystems, provided that roots are extant and relative humidity is high (Hoch 1994, 2002). These roots, usually developed in the topsoil and the MSS, can develop deeper, emerging in the caves from cracks forming long strands hanging from the ceiling of the cave, or form a fine root network running

over the walls and floor of the cave (Fig. 2) such as in limestone caves, lava tubes, and also in granitic chaos (Table 1). As strictly phytophagous insects, the cave-dwelling planthoppers are primary consumers in the subterranean ecosystems (Hoch and Howarth 1993).

Knowledge of cave-dwelling planthoppers remains generally limited to the description of the species. Much of what we know about the biology of cavernicolous planthoppers comes from a single case study on the blind, flight- and pigmentless *Oliarus polyphemus* Fennah from Hawaii Island (Hoch and Howarth 1993). Field observations on various aspects of the biology of other species are still scarce (e.g., Hoch and Asche 1993; Hoch et al. 2006, 2014, 2021; Soulier-Perkins et al. 2015; Le Cesne et al. 2022, 2023).

The roots system of the plants provides them with a relatively abundant food but limited by an epigean flora developing long roots, which however confine them to the environment of shallow caves. These roots are also an ideal medium to communicate with the other individuals, in particular to meet mating partners as in an epigean life. Indeed, as with their epigean relatives (Claridge 1985), cave planthoppers produce low-frequency, substrate-borne vibrations to communicate. This behavior, which is general in planthoppers (Soulier-Perkins et al. 2015) and relies on this communication channel for their mating behavior (Hoch 2002), has been confirmed and analyzed (*in-* and *ex-situ*) on several occasions with these cavernicolous species (Hoch and Howarth 1989b; Hoch and Howarth 1999; Hoch and Wessel 2006; Soulier-Perkins et al. 2015). As with epigean species (Hoch 2002; Soulier-Perkins et al. 2015), it has been suggested that this behavior would allow them to locate a potential mate also in the permanent darkness of caves. However, as with epigean species, one cannot exclude other behaviors such as possible territorial rivalry between males (Soulier-Perkins et al. 2015).

While the eyes of adult cave-dwelling species are often reduced or absent, the antenna remains well developed, especially with the characteristic large olfactory placoid sensilla on the pedicel in planthoppers (Hoch et al. 2006; Santos et al. 2023). As already shown in several epigean species (Aljunid and Anderson 1983; Young 2002; Riolo et al. 2012), their role in the search for food roots via plant volatile compounds is likely, while their role for other intraspecific interactions, possibly pheromone-driven, cannot be ruled out (Wang et al. 2018), although experimental evidence is lacking.

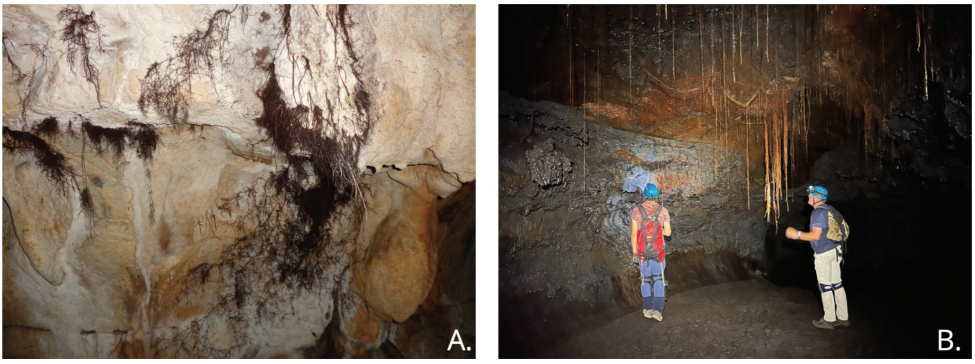

Figure 2. A roots along the wall in a limestone cave of the south of France (Grégoire Maniel) **B** roots hanging from the ceiling of a lava tube in La Réunion (Fred Melon).

Reproduction, life history and postembryonic development

It has been observed that females of the troglobitic *Oliarus polyphemus* from Hawaii lay very few eggs, suggesting a low reproduction rate, a typical K-selection process found in ecologically stable environments (Hoch and Howarth 1993) and documented for many obligate cave species (Culver 1982). *Oliarus'* eggs are deposited in a wax-filament nest on roots. The nymphs are usually found close to the roots while the adults are generally active and found throughout the cave (Hoch and Howarth 1993). In contrast, adults of *Coframalaxius* Bourgoin & Le Cesne, 2022, from Southern France were found together inside the waxy nests, little active, while nymphs were found active throughout the cave, close to other roots (Le Cesne et al. 2022). In *Typhlobrixia* Synave, 1953, both nymphs and adults were observed in isolation within the Tsingy Namoroka cave system in Madagascar, indicating that both are potential dispersal stages. However, adults were also frequently encountered in close proximity to roots (Soulier-Perkins et al. 2015).

Although the nymphal morphology even of epigean Cixiidae is not well-documented, it is reported that their first instars have very low pigmentation and are either blind or possess only a few ommatidia. The development of their compound eyes begins only after the third or fourth instar (Wilson and Tsai 1982; Wilson et al. 1983; personal observation of the authors). Although a comprehensive description of all nymphal instars of the troglobiont kinnarid *V. fadaforesta* Hoch & Sendra, 2021 has been recently published (Ortega-Gomez et al. 2022), which reports the absence of eyes and ocelli since the first instar, the nymphal morphology of epigean Meenoplidae-Kinnaridae nymphs remains unknown, a fact which impairs a direct comparison.

Colonisation and evolution

Two hypotheses have been proposed to explain the evolution of cavernicoly. The "climatic relict hypothesis" (CRH) was initially proposed by Vandel in 1964 (Barr 1968), and further developed by Peck and Finston (1993). It suggests that the presence of troglobionts can be attributed to past changes in epigean abiotic factors, such as climatic changes, which constrained epigean species and driven them to colonize subterranean habitats as refuges. According to this hypothesis, it is expected that the insects that colonized subterranean habitats do not have any extant close relatives today (Fig. 3, CRH), as those close relatives were unable to adapt to the changes in epigean abiotic factors or are at least allopatrically distributed compared to the cave-dwelling species (Wessel et al. 2007).

On the other hand, Howarth (1980, 1983) proposed the "adaptive shift hypothesis" (ASH), which suggests that cavernicolous animals are present in suitable subterranean areas due to active colonization of subterranean habitats as new niches through "adaptive shifts" of epigean species. According to this hypothesis, one would expect to observe hypogeal species that are closely related to their epigean counterparts in a parapatric distribution (Wessel et al. 2007) (Fig. 3, ASH).

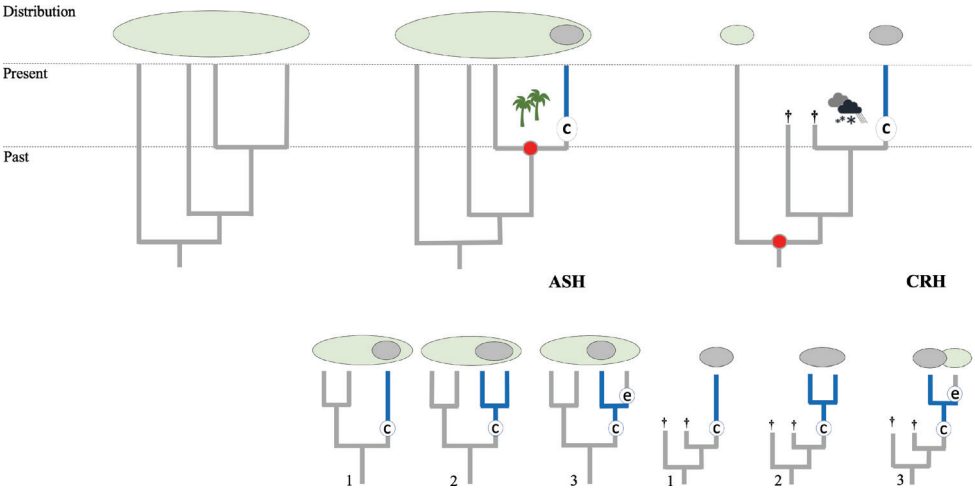

Figure 3. Resulting distributions and phylogenies of closely related species with one species moved to cavernicoly (C), according the two explanatory models, the 'Adaptive Shift Hypothesis' (ASH) or the 'Climatic Relict Hypothesis' (CRH). with possible subsequent scenarios: in-cave speciation (ASH 2, CRH 2) or possible return to epigean (E) conditions (ASH 3, CRH 3). Red circle denotes the node of the first common ancestor linking the cave species and its closest extant epigean relative.

To determine which of the two explanatory models applies in a given case, Wessel & al (2007) suggested that a phylogenetic analysis of the faunas should be undertaken: an allopatric or parapatric speciation will respectively accredit the "climatic relict" or "adaptive shift" model as a possible speciation process explanatory hypothesis (Fig. 3).

Why do cave planthopper studies matter?

Planthoppers in light of definitions: the limits of morphological and etho-ecological classifications

The first classification of cave organisms was based on their degree of morphological adaptation to the hypogean habitats (Shiner 1854) and adapted by Racovitza in 1907 who recognized three categories: the trogloxenes (temporary visitors to caves), the troglophiles (facultatively cavernicolous) and the troglobionts (obligately cave-dwelling species). However, the transitional category troglophile has always been difficult to define. Reviewing the century of evolution of the subterranean organism's classification, Sket (2008) proposed an ecology-based terminology. Accordingly, subterranean species are now standardly classified as true cavernicolous or troglobionts (species strictly bound to the hypogean habitats), eutroglophiles (epigean species able to maintain permanent hypogean populations), subtroglophiles (epigean species living temporally or cyclically during their life in hypogean conditions) and trogloxenes (species occurring sporadically in a hypogean habitats, unable to establish subterranean stable populations) (Sket 2008; Howarth and Moldovan 2018b).

Just as the degree of troglomorphy appeared to be a criterion difficult to apply for classifying subterranean organisms, Wessel et al. (2007) and Hoch et al. (2014) have shown that it is neither a reliable indicator for the age of the cavernicolous lineage, which in Hawaii and Australia for instance, does not necessarily correlate with the age of cave. Moreover, if cave-adaptation and troglomorphies are strongly linked to the troglobiont category of Sket (2008), the opposite is not true and a troglobiont species does not necessarily exhibit troglomorphies. Indeed, it might be not that common, but some species found in caves without troglomorphies although never found at the surface, might have been called trogloxene when they might be true troglobiont or at least eutroglophile as pointed out by Deharveng et al. (2022).

From Sket's 2008 etho-ecological perspective, and for obligatory phytophagous insects such as planthoppers, the root system of the epigean vegetation offers the opportunity to access the underground environment in temporary, cyclical or even permanent hypogean conditions. In Cixiidae for instance, the nymphs of most if not all epigean species live underground: should we consider them as subtroglophile species living cyclically during their life in hypogean conditions? e.g. does Cixiidae (as well as Meenoplidae-Kinaridae) be considered as a subtroglophile taxa, a subtroglophile family rank lineage?

Although the Sket's 2008 new classification represents a progress in better classifying undergrounds organisms offering a more precise and less arbitrary grouping system, it still leaves place to some ambiguities (Howarth and Moldovan 2018b). Neither do Sket's 2008 ecological categories constitute an evolutionary gradient pointing towards a fully adapted cave-dwelling species.

Moreover, with time during its evolutionary history, each species continues their evolution according to the ecological opportunities of its immediate environment and to adapt towards new epigean, hypogean or mixed environments. Cave adaptation is not a dead-end road of evolution. A well-studied example of such subterranean speciation exists in the Hawaiian cave planthopper *Oliarus polyphemus*. It has been demonstrated that morphologically similar, yet behaviorally distinct populations of this blind, unpigmented and flightless taxon from lava tubes on the Big Island of Hawaii, in fact are a complex of at least 12 closely related, but reproductively isolated species (Hoch and Howarth 1993; Wessel et al. 2013). Most likely they are the result of a non-adaptive radiation triggered by the rapid vegetational succession on active volcanoes.

In contrast, a true troglobiont population might also be able to evolve again into a surface-dwelling species if conditions permit, as has been described for crickets (Desutter-Grandcolas 1993: fig. 2).

In summary, it can be stated that, the degree of troglomorphy is not indicative of a phylogenetically older lineage, nor does it necessarily express a *per se* adaptation to hypogean life, nor is troglobiosis an evolutionary dead end of an evolutionary lineage.

Instead, the degree of troglomorphy has been shown to correlate with the special conditions of the environment (Hoch and Howarth 1989a, b). In the Australian cixiid genus *Solonaima*, four separate independent cave invasions have been documented from lime stone caves and lava tubes in Queensland (Hoch and Howarth 1989b). The cavernicolous *Solonaima* species display varying degrees troglomorphy, ranging from mild eye-, pigmen-

tation-, and wing reduction to the partial or entire loss of compound eyes, pigmentation and wings. Ages of caves range from 190 000 year-old lava tubes (Undara) to 5 million year-old limestone caves (Chillagoe Karst). Interestingly, the least modified (facultative) cave species occur in the geologically oldest, most eroded and comparatively open caves, those with intermediate degrees of troglomorphy in deeper caves, and while the most highly modified species, *Solonaima baylissa* Hoch & Howarth, 1989, is restricted to damp passages with high CO_2 levels in the deep cave zone of the younger lava tubes in Undara.

Whether based on morphology or etho-ecology, these classification systems remain imperfect (Howarth and Moldovan 2018b). In addition, they only take into account morphologies or life traits that have already been achieved for adaptation to troglodytic life, a way of life that could have started well before. How can we better take into account this "elusive" period from a morphological and ecological point of view? The physiological adaptations of organisms to obligately cavernicolous life probably precede the completed morphological and ecological transformations that we observe. These adaptations are also diverse, probably not all concomitant, nor necessarily biologically linked at the start: adaptations to small variations in temperature, to 'warm' tropical caves or 'cold' ones in temperate environments, to the absence of circadian rhythm, to the absence of light, to high humidity, to scarcity of resources, etc. Trying to integrate them into the classification system of cave organisms and that of their type of environment (Howarth and Moldovan 2018a, b), remains a major challenge to better understand and more precisely analyze the drivers of cavernicoly.

Planthoppers, cavernicoly and evolutionary processes: the limits of the two hypotheses

In theory the two scenarios proposed by Vandel (1964) and Howarth (1980) could logically explain the observed distributions of cave species and their closest related taxa. For instance, the active speciation highlighted by Wessel et al. (2013) of the cavernicolous *Oliarus* species of the young Hawaiian lava tube system rather fits the criteria of an adaptive shift (Howarth 1980, 1983). On the contrary, the recently described kinnarid *Valenciolenda* species Hoch & Sendra, 2021 from Spain (Hoch et al. 2021), being the only species of this family from the western continental Palearctic, would suggest a pattern of distribution that would fit with a speciation process following the relict hypothesis of Vandel (1964).

However, this may have been more complicated in reality, where several events may have taken place between the time of the first evolution of a species to cavernicoly and the current observation of the distributions of the closely related lineages. What can happen once an organism has adapted to underground habitats? 1) it can continue to diversify in the underground environment and new speciations take place (Hoch and Howarth 1993; Wessel et al. 2013; Huang 2022) (Fig. 3 ASH2 and CRH2), or 2) it can continue to diversify and might recolonize above ground habitats (Desutter-Grandcolas 1993) (Fig. 3 ASH3 and CRH3). With such possible scenarios Fig. 3 shows that the observed distributions would not be sufficient alone to discriminate between the different possible scenarios.

It should be noted that the climatic relict hypothesis and the adaptive shift hypothesis are not mutually exclusive. From a theoretical point of view, however, a clear distinction must be made between pattern (distribution) and process (factors driving speciation). The distribution patterns we see today must not necessarily reflect the processes which favored adaptations to novel environments such as subterranean habitats, e.g., MSS or caves. It is conceivable that in a given biotope cave adaptation through an adaptive shift could be followed by totally independent severe climatic constraints that would eliminate the related epigean species. Such a scenario could also bias distribution observations and would mistakenly favor the relict model as the selected process to explain the pattern observed. Even if past cave colonization events could be correlated by calibrated phylogenies with certain major known climatic events (e.g. past glaciations in Europe), a causal determination cannot *a priori* be assumed.

Both hypotheses have merely – even if limited – explanatory power to reconstruct the evolutionary scenario(s) under which cave adaptation may have occurred in each specific case.

Why live underground? From exaptation to adaptation

Subterranean life has played a significant role in shaping its inhabitants through evolutionary trends most often characterized by reduction, which has been studied since the earliest observations of cavernicolous animals (Racovitza 1907; Barr 1968; Culver 1982; Gibert and Deharveng 2002; Romero 2009; Mammola 2019, etc.). Troglomorphic insects, specifically, are known for exhibiting notable reductions in pigmentation, eye and wing sizes in adult individuals (Culver and Pipan 2018), but also specializations including elongated appendages, the development of specialized sensory organs, and an extended lifespan (Hoch 1994; Hoch 2002). Indeed, while most apparent traits for subterranean planthoppers involve such reduction or loss of certain morphological characteristics compared to their adult epygean relatives, other features with presumed increased adaptive value have also been suggested (Wessel et al. 2007). For example, Howarth (1981) described a specialized spine configuration on the tarsi of Hawaiian cavernicolous species of *Oliarus*, to enhance the insect's ability to walk on wet rocky surfaces. Another example is the Malagasy species *Tsingya clarkei* Hoch & Wessel, 2014, which exhibits a potential case of insular gigantism or autapomorphic giantism (Gould and MacFadden 2004) with the size of the species being more than twice larger than the other species of the family. However, in that specific case more than due to cavernicoly adaptation, the authors hypothesized this specialization as the possible result of a relaxation of predation and competition pressures together with random genetic drift (Keogh et al. 2005; Hoch et al. 2014).

Another interesting specialized pattern observed in several cave planthoppers is the heightened activity of the tegumentary gland system, responsible for wax production. These wax glands are also found in larger quantities, particularly in the tegmina and peripheral membrane of species such as *Valenciolenda fadaforesta*, *Solonaima baylissa*, *Ibleocixius dunae*, and *Typhlobrixia namorokensis* Synave, 1952. The hypertrophy of the glandular system (Hoch 2002) and increased complexity of chemosensory systems

(Balart-Garcia et al. 2022) could be correlated with the enhanced sensory function often observed in cave-dwelling organisms.

From a physiological perspective, cave planthoppers have undergone adaptations that render them indifferent to significant circadian and direct seasonal fluctuations, much like other true troglobitic species (Howarth and Moldovan 2018b). In turn, they have adapted to the absence of light, high and stable humidity levels, constant temperatures, and a scarcity of food resources restricted to the presence of roots in caves. As a result, these species have developed effective dispersal behaviors for locating food resources, relying more on walking than flying in total darkness. However, in open spaces, they sometimes exhibit a unique avoidance behavior resembling a parachute escape, where they jump followed by a gliding flight without wing beats, as observed in the recently discovered Spanish species *Valenciolenda fadaforesta*, and in the Australian cixiid species *Solonaima baylissa*, found in Queensland lava tubes (Hoch and Howarth 1989b). This behavior described as "parachuting", allows them to evade predators without straying too far from their rare and valuable food sources (Hoch et al. 2021).

However, while direct influences of seasonal fluctuations are excluded, there are slight and gradual indirect modifications of temperature and humidity that still regulate the seasonal distribution of insects within the MSS (Mesovoid Shallow Substratum) and floodable spaces. Moreover, the seasonal physiology of epigean plants through their roots might also influence seasonal patterns in the biology of planthoppers in an environment that still presents low seasonal fluctuations and is not completely stable (Lawton and Lawton 1971; Furukawa et al. 2011; Miller et al. 2020; Losso et al. 2023).

Alongside reporting morphological and physiological adaptations to underground life, it was assumed that subterranean environment was too "harsh" to be colonized without any preadaptation of its colonizers (Christiansen 1992; Holsinger 2000). On this same line, several authors (Oromí 2004; Giachino and Vailati 2005, 2006, 2010, 2016; Moseley 2009; Monguzzi 2011; reviewed in Mammola et al. 2016), have suggested that MSS should be regarded as the primary habitat for the subterranean fauna, a first step to the colonization to the MSP. Accordingly, MSS should be considered 'part of the hypogean ecosystem and represents its extension toward the surface' (Mammola et al. 2016). Romero (2009), however, questioned this notion of preadaptation ‹accepted without question' in biospeleogical publications, and showed that characters, supposed to be "pre-adaptations", in related fish taxa for instance, are not statistically significant to conclude that they were the main driver to cave colonization. From a theorical perspective, Gould and Vrba (1982) developed the concept of exaptation as an opportunistic selective adaptation, favoring traits that would become useful for a new function, for which they were not initially selected. Some planthopper troglomorphies have been considered as exaptations that likely facilitate subterranean colonization by organisms (Hoch 2002; d'Urso and Grasso 2009).

Contrasting evolutionary patterns in planthoppers: the Cixiidae case

With a few exceptions, only two main independent lineages, the Cixiidae and the Meenoplidae-Kinnaridae, have successfully colonized underground ecosystems. These

lineages are considered groups of epigean species whose larval instars are well known to feed on roots (Hoch 2002; Wessel et al. 2007; Bowser 2014; Bartlett et al. 2018). Both lineages exhibit exaptations in their larval instars for root feeding in the interstitial environment, enabling them to completely switch entirely to an endogeic life. However, the collected data reveal contrasting patterns: 50% of the described cave species belong to the Cixiidae, with 44 species out of more than 2600 species (1.6%), while the Meenoplidae-Kinnaridae comprise 21 species out of 285 species (7.3%) (Bourgoin 2023b). These patterns raise some interesting questions:

Why do other planthopper taxa with similar behavior and ecology, such as Tettigometridae, which are well-known root feeders and are often tended by ants underground (Bourgoin et al. 2023b), lack troglobiont representatives? Did Tettigometrids fail to undergo the necessary adaptations to thrive in subterranean environments? Could it be that the availability of suitable resources, physiological adaptations, and dispersal capabilities did not align within this particular lineage? Why are there so few cave-dwelling Delphacidae, or Derbidae or Achilidae although the nymphs of many species live close to the soil? Even more generally, why are there no cavernicolous Cicadomorpha at all? At least in the Cicadoidea (Cicadidae and Tettigarctidae) and Cercopidae one should expect some, as their nymphs also live underground, feeding on roots (Strümpel 2010, and references therein).

The presence of blind and unpigmented cixiid nymphs feeding on subterranean roots could indeed be considered as a potential exaptation, providing a foundation for the evolution of complete subterranean life. The cryptic, or even subterranean lifestyle of their nymphs is probably a specific trait of the family Cixiidae (Asche 1988). Could we consider Cixiidae as a subtroglophile lineage? Why then, however, are there only 44 species of Cixiidae which have successfully made the shift to cavernicoly? Which are the additional factors at play, beyond the initial exaptation, that determine or block the colonization and persistence of species in subterranean habitats? An example is the cixiid genus *Hyalesthes* Signoret, 1865 from the Canary Islands which is represented with several only epigean species throughout the archipelago (Hoch and Remane 1985). Adult *Hyalesthes* individuals are frequently observed in caves, however, apparently without establishing permanent subterranean populations (Hoch, unpublished). In contrast, other cixiid taxa, *Cixius* Latreille, 1804 and *Tachycixius* Wagner, 1939, while rare in surface habitats, have brought forth several separate lineages which have adapted to the subterranean biome (Hoch and Asche 1993).

How physiological adaptations specific to subterranean life, such as modifications in sensory systems, metabolism, or reproductive strategies of the different cixiid lineages, may also play a crucial role in successful colonization? Additionally, how the ability to disperse and establish populations in subterranean environments may have been influenced by dispersal capabilities, geographic barriers, or interactions with other organisms in the underground ecosystem?

While in theory, any cixiid species could potentially undergo an adaptive shift and make the transition to an entirely subterranean lifestyle, it is essential to critically analyze how exaptations take place: special morphological or behavioral traits might be necessary or not or but not sufficient in determining the success or failure of species in colonizing subterranean habitats.

Conclusions

According to the bioclimatic model proposed by Howarth (1980), terrestrial troglobites, can be expected "in any region which is old enough and in which there are extensive caves with an adequate moisture supply and a continuous equitable environment and food supply for colonization" (Howarth 1980: 403). Cave-dwelling planthoppers are found in many parts of the world (Fig. 1), and new species are continuously being discovered. Their occurrence is not limited to the current occurrence of related epigean taxa. They can thus shed light on past distribution of their lineages, leading to interesting questions of biogeography, regardless of climatic changes.

Planthoppers are a highly diverse taxon, occurring in a wide variety of habitats and climatic zones. This makes them ideal models for the study of troglobiont evolution. Comparative studies of nymphal morphology, biology and behavior of cixiids and meenoplidkinnarids, the latter being virtually unknown, may provide deeper insights in the ground pattern of Fulgoromorpha and eventually, a more complete picture of the factors leading to the evolution of troglobiont taxa. Specifically, the singularity of cavernicolous lineages within otherwise epigean clades (e.g, the genus *Notuchus* with 3 troglobiont species, within the Delphacidae, *Budginmaya eulae* within the Flatidae) and the phylogenetically isolated *Hypochthonella caeca* (being the only species of the Hypochtonellidae), deserves to be studied in depth, particularly from a phylogenetical perspective.

Apart from the evolutionary point of view, the existence of cavernicolous taxa raises attention to issues of conservation. Underground habitats are characterized by environmental stability, high humidity, and darkness (Poulson and White 1969; Culver 1982; Souza Silva et al. 2015; Sanchez-Fernandez et al. 2018). The inhabitants of these ecosystems are highly adapted to these conditions, and any disturbances can have detrimental effects on the associated fauna. Culver and Pipan (2010) demonstrated how abiotic changes in surface ecosystems can lead to the colonization of subterranean habitats. However, the indirect impacts of these changes on the underground world can be "fatal" to the existing cave inhabitants (Trajano 2000). The causes of such changes can be diverse, and many human activities have implications for the subterranean realm (Ferreira and Horta 2001; Faille et al. 2015b; Monro et al. 2018; Costa Cardoso et al. 2021). In the actual context of a biodiversity crisis, the question of the conservation of these ecosystems and their inhabitants is a priority. In line with this reflection, a cave conservation index of priority has been suggested (Souza Silva & al., 2015) and a roadmap to follow (Wynne et al. 2021). The cave-dwelling planthoppers are no exception, and already new conservation status have been published for some species (Santos et al. 2018; Borges et al. 2019; Hoch et al. 2021). Such actions will allow to better preserve the subterranean biome and collect data on their specialized, endemic and narrow range faunas, including planthoppers and other invertebrates.

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Professor Boris Sket (1936–2023): the SpeleoBiologist and much more

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Professor Boris Sket (Fig. 1) passed away on May 7, 2023, in Ljubljana at age 86. Many of us will remember him as a great naturalist, with a broad interest in zoology, botany, evolution, ecology, biogeography, and his main passion: subterranean fauna. Ashes to ashes; however, his research legacy remains. It is our honor to look back on his life and work to say goodbye.

To truly grasp and appreciate his nearly seven decades of research, we must journey back to the aftermath of the Second World War, when the map of Europe was redrawn. The Kingdom of Yugoslavia underwent a formal transformation into the Socialist Federative Republic of Yugoslavia led by communist party. Although the Yugoslavian communist regime cannot compare to much harsher communist rulers in countries of Eastern Europe, the nation faced economic challenges, less porous borders, and greater difficulty in communicating with the outside world compared to today. Nonetheless, Yugoslavia encompassed almost the entire Dinaric Karst, nowadays well known as a global hotspot of subterranean biodiversity. Exploration of caves was at that time already established and much of this exploration was led by biologists (Sket 2008a); however, large parts of the territory were unexplored at that time and much of the fauna was unknown.

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Figure 1. Boris Sket, 1936–2023 (Photo: Boris Sket archive).

Young Boris became acquainted with subterranean fauna early, amidst the turmoil of war, when Ljubljana was occupied and surrounded by barbed wires. During that period, he frequented the National Museum in Ljubljana (today Slovenian Museum of Natural History), where his attention was captured by two preserved specimens of subterranean crustaceans of the genera *Niphargus* and *Troglocaris*. Immediately following the war, he embarked on cycling expeditions with his grandfather to smaller caves in the vicinity of Ljubljana, where he was fascinated by whitish animals, most likely *Niphargus*. His early education was marked by frequent relocations between Ljubljana and Belgrade. Boris' professional journey into the exploration of subterranean life truly began with his enrollment at the University of Ljubljana, and his subsequent role as a teaching assistant to Professor Janez Matjašič (Box 1, Fig. 2).

The fieldwork activities at that time were far more time-consuming and relied heavily on improvisation compared with modern practices. Transportation options were limited, and commonly relied on trains and bicycles. The caving equipment was less sophisticated and under the development: the clothing resembled that of mountaineers, carbide flames preceded the powerful LED lamps of today, ladders ruled before ropes, homemade masks, pipes and air pumps were utilized before the advent of scuba diving gear. Nets, sieves, and filters were crafted at home with the assistance of his technical assistant, the malacologist and close friend France Velkovrh. One of Boris's most notable

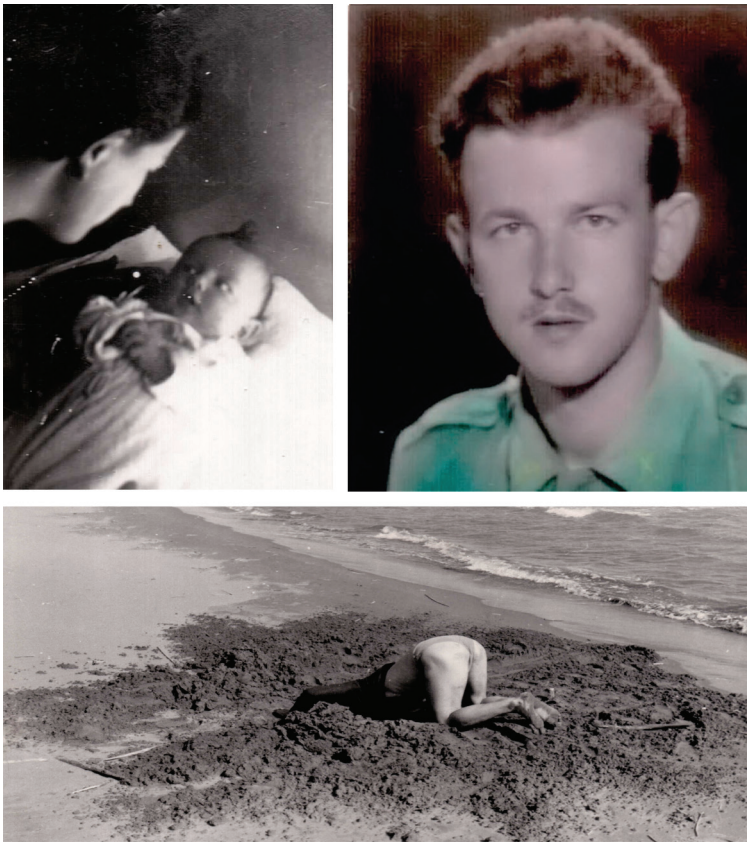

Figure 2. Timeline of Boris Sket. Upper left: just arrived in 1936. Upper right: serving army near Valjevo, Serbia. Bottom: sampling interstitial near Ulcinj, Montenegro. (Photo: Boris Sket archive).

inventions was the so called "Sket bottle" (Chevaldonné et al. 2008; Sket 2018), a cleverly designed plastic bottle used for animal suction during snorkeling or diving. This period of field work importantly contributed to the accumulation of empirical evidence that the subterranean environment extends beyond caves, encompassing both the fissure systems of consolidated cracked carbonate rock and unconsolidated riverine sediments.

The Yugoslav regime was supporting nature exploration, although there was no imperative for publishing results. Much of Boris' research results from that period was documented in what is now termed "grey literature". Some of his findings were disseminated at international conferences and subsequently published in conference proceedings. Reports of his studies were submitted in multiple copies to funding authorities in Slovene language. Despite the absence of pressure to publish internationally, Boris' research opus is impressive, both in terms of breadth and depth. His attitude toward life and work can be summarized in three words: passion, curiosity, and persistence. He was a biologist *par excellence*, a researcher of numerous interests, ranging from pure nature observation through the lens of a camera to taxonomy, ecology, evolution, and

Box 1. The academic career of Boris Sket.

Education:

1961: PhD thesis entitled "Specialization of our freshwater isopods"; University program in Ljubljana (at that time named as Prirodoslovno-matematična fakulteta)

Employment:

1959–1969: Teaching assistant of professor Janez Matjašič.
1969–1974: Assistant professor for the zoology and speleobiology.
1974–2005: Full professor for the zoology and speleobiology
2005–2013: Researcher.
2013–2023: Retired yet active.

Mentorship:

10 PhD students
5 MSc students
25 Graduate students

Professional activities at the University:

1974–2012: Leader of the Research group for invertebrate zoology and speleobiology
1981–1983: Vice-dean of the Biotechnical Faculty at the University of Ljubljana.
1983–1985: Dean of the Biotechnical Faculty at the University of Ljubljana.
1985–1987: Vice-dean of the Biotechnical Faculty at the University of Ljubljana.
1989–1991: Rector of the University of Ljubljana.

Other professional activities:

1976–1980: President of Caving Society of Slovenia.
1998–2011: Leader of research Program "Zoology and speleobiology" (P1–0184, funded by Slovenian Research Agency).
2004–2008: President of the International Society for Subterranean Biology.
2011–2023: A member of Slovenian Academy of Science and Arts
2009–2023: A member of Bosnian Academy of Science and Arts

Awards:

Student's award of France Prešern.
1965: Award of Boris Kidrič.
1979: Order of labor, third class (silver wreath).
1988: Honorary member of Yugoslavian Cave association.
1991: Order of republic, third class (silver wreath).
1995: Jesenko Award of Biotechnical Faculty of University of Ljubljana.
2003: Zois Award for scientific excellence.
2008: Golden award of Slovenian Caving Association.
2010: Award for life work of Miroslav Zei of the National Institute of Biology.
2016: Honorary membership of the International Society of Speleobiology.

Editorial activities:

1997–2022: Acta Biologica Slovenica
2002–2023: Subterranean Biology
2005–2023: Zootaxa

Research output:

Over 350 articles
Over 570 bibliographic units (source: Slovenian bibliographic database)

conservation. While he was interested in both surface and subterranean life, the majority of his research efforts were dedicated to the latter. Over his long and fruitful life, he witnessed the transformation of the Dinaric Karst: from the pristine natural state during the pioneering years, to expanding urbanization, river channelization, damming and general degradation of the environment.

His contribution to science is impressive. Sometimes, he acted as the leading researcher, sometimes as a provider of data and ideas, and sometimes as a knowledgeable peer with immense field experience. Above all, he was a dedicated teacher who passed on his knowledge to successive generations of biologists of all formats and specializations. Here, we broadly expose his contributions to science, including work of his collaborators and students. We divided his opus into five arbitrary topic sections and a box summarizing his academic career.

Taxonomy & natural history

Many researchers will remember Boris as a taxonomist of broad interests, delving into the taxonomy of species across numerous phyla. According to his own words, his taxonomic expertise was "an unwanted need rather than his primary research interest" on a way to comprehend the subterranean Dinaric fauna. Taxonomic descriptions were the *sine qua non* for the rest of his work. Indeed, many discoveries of Dinaric species can be attributed to his research efforts.

Boris advocated a pragmatic use of the biological species concept, wherein morphological characters served as hints to potential or actual reproductive barriers. He maintained skepticism towards the uncritical usage of the term "cryptic species" asserting that genuine morphological crypticity could not be reliably distinguished from inadequate morphological examination. He was playful and humorous in naming new species: the black olm was named "parkelj", which is the Slovenian name of a traditional infernal figure accompanying St. Nicholas. Black with a red tongue, it resembles the black and red color combination in the non-troglomorphic morph of the olm. His main taxonomical contributions are outlined in five subsections, and the list of taxa he described is available in Table 1.

Annelida: Clitellata

Findings of leeches in Dinaric caves had two important consequences for Boris. First, he became one of the few global authorities in leech taxonomy, who curated several regional and global leech checklists (Sket 1986c; Sket and Trontelj 2008; Minelli et al. 2014), and served as a subject editor at Zootaxa. He studied distribution, ecology and taxonomy of glossiphoniid (Sket 1968), piscicolid (Sket 1985b) and several erpobdellid leeches (Sket 1968, 1981c; Sket and Šapkarev 1986; Sket 1989, 1992b), including the peculiar *Croatobrancus mestrovi* from deep caves of Velebit (Sket et al. 2001).

Table 1. List of taxa described by Boris Sket. Families and genera are described with G and F, respectively.

Classification	Taxon described	Category
Porifera		
Spongillidae	*Eunapius subterraneus* Sket & Velikonja, 1984	
Cnidaria		
Hydrozoa:	***Velkovrhia*** Matjašič & Sket, 1971	**G**
Bougainvilliidae	*Velkovrhia enigmatica* Matjašič & Sket, 1971	
Annelida: Clitellata: Rhynchobdellida		
Erpobdellidae	*Dina eturpshem* Sket, 1989	
	Dina krasensis (Sket, 1968)	
	Dina krilata Sket, 1989	
	Dina lepinja Sket & Šapkarev, 1986	
	Dina dinarica Sket, 1969	
	Dina lacustris Sket, 1970	
	Dina montana Sket, 1971	
	Dina ohridana Sket, 1968	
	Dina svilesta Sket, 1989	
	Trocheta dalmatina Sket, 1968	
Glossiphoniidae	*Glossiphonia complanata maculosa* Sket, 1968	
	Glossiphonia pulchella (Sket, 1968)	
Piscicolidae	*Cystobranchus pawlowskii* Sket, 1968	
	Piscicola hadzii Sket, 1985	
Arthopoda: Malacostraca		
Decapoda		
Atyidae	***Ficticaris*** Sket & Zakšek in Jugovic, Zakšek, Petković & Sket, 2019	**G**
	Ficticaris serbica Jugovic & Sket in Jugovic, Zakšek, Petković & Sket, 2019	
	Gallocaris Sket & Zakšek, 2009	**G**
	Spelaeocaris kapelana Sket & Zakšek, 2009	
	Spelaeocaris neglecta Sket & Zakšek, 2009	
	Spelaeocaris prasence Sket & Zakšek, 2009	
	Troglocaris anophthalmus legovici Jugovic, Jalžić, Prevorčnik & Sket, 2012	
	Troglocaris anophthalmus ocellata Jugovic, Jalžić, Prevorčnik & Sket, 2012	
	Troglocaris anophthalmus periadriatica Jugovic, Jalžić, Prevorčnik & Sket, 2012	
	Troglocaris anophthalmus sontica Jugovic, Jalžić, Prevorčnik & Sket, 2012	
	Troglocaris bosnica Sket & Zakšek, 2009	
Gecarcinucidae	*Sundathelphusa boex* Ng & Sket, 1996	
	Sundathelphusa sottoae Ng & Sket, 1996	
	Sundathelphusa urichi Ng & Sket, 1996	
	Sundathelphusa vedeniki Ng & Sket, 1996	
Amphipoda		
Anisogammaridae	***Fuxiana* Sket, 2000**	***G***
	Fuxiana yangi Sket, 2000	
	***Fuxigammarus* Sket & Fišer, 2009**	***G***
	Fuxigammarus antespinosus Sket & Fišer, 2009	
	Fuxigammarus barbatus Sket & Fišer, 2009	
	Fuxigammarus cornutus Sket & Fišer, 2009	
Bogidiellidae	*Bermudagidiella bermudiensis* (Stock, Sket & Iliffe, 1987)	
	Bogidiella gammariformis Sket, 1985	
	Bogidiella sinica Karaman & Sket, 1990	
Gammaridae	***Dinarogammarus* Sket & Hou, 2018**	***G***
	***Relictogammarus* Hou & Sket, 2016**	***G***
	***Iberogammarus* Sket & Hou, 2018**	***G***
	Gammarus parvioculatus Sidorov, Hou & Sket, 2018	
	Gammarus troglomorphus Sidorov, Hou & Sket, 2018	
	Neogammarus gordankaramani (Özbek & Sket, 2020)	

Classification	Taxon described	Category
Crangonyctidae	*Tadzocrangonyx alaicus* Sidorov, Hou & Sket, 2018	
Melitidae	*Melita mirzajanii* Krapp-Schickel & Sket, 2015	
Niphargidae	***Carinurella* Sket, 1971**	***G***
	Carinurella paradoxa (Sket, 1964)	
	***Chaetoniphargus* Karaman G.S. & Sket, 2019**	***G***
	Chaetoniphargus lubuskensis Karaman G.S. & Sket, 2019	
	***Niphargobates* Sket, 1981**	***G***
	Niphargobates orophobata Sket, 1981	
	Niphargobatoides lefkodemonaki (Sket, 1990)	
	Niphargus aberrans Sket, 1972	
	Niphargus brevirostris Sket, 1971	
	Niphargus carniolicus Sket, 1960	
	Niphargus dabarensis Fišer, Trontelj & Sket, 2006	
	Niphargus dobati Sket, 1999	
	Niphargus factor Sket & G. Karaman, 1990	
	Niphargus jadranko Sket & G. Karaman, 1990	
	Niphargus labacensis Sket, 1957	
	Niphargus liburnicus G. Karaman & Sket, 1989	
	Niphargus lourensis Fišer, Trontelj & Sket, 2006	
	Niphargus microcerberus Sket, 1972	
	Niphargus minor Sket, 1957	
	Niphargus multipennatus Sket, 1957	
	Niphargus numerus G. Karaman & Sket, 1990	
	Niphargus pachytelson Sket, 1960	
	Niphargus pectencoronatae Sket & G. Karaman, 1990	
	Niphargus pectinicauda Sket, 1971	
	Niphargus polymorphus Fišer, Trontelj & Sket, 2006	
	Niphargus pretneri Sket, 1959	
	Niphargus pupetta (Sket, 1962)	
	Niphargus rejici Sket, 1958	
	Niphargus rostratus Sket, 1971	
	Niphargus scopicauda Fišer, Coleman, Zagmajster, Zwittnig, Gerecke & Sket, 2010	
	Niphargus stenopus Sket, 1960	
	Niphargus subtypicus Sket, 1960	
	Niphargus transitivus Sket, 1971	
	Niphargus trullipes Sket, 1958	
	Niphargus vinodolensis Fišer, Sket & Stoch, 2006	
Pseudoniphargidae	*Pseudoniphargus carpalis* Stock, Holsinger, Sket & Iliffe, 1986	
	Pseudoniphargus grandimanus Stock, Holsinger, Sket & Iliffe, 1986	
Seborgiidae	*Seborgia kanaka* Jaume, Sket & Boxshall, 2009	
	Seborgia sanctensis Jaume, Sket & Boxshall, 2009	
	Seborgia vietnamica Jaume, Sket & Boxshall, 2009	
Ingolfiellidae	*Ingolfiella* (*Tethydiella*) *longipes* Stock, Sket & Iliffe, 1987	
Isopoda		
Anthuridae	*Stygocyathura filipinica* (Botosaneanu & Sket, 1999)	
Asellidae	*Asellus* (*Asellus*) *aquaticus carniolicus* Sket, 1965	
	Asellus (*Asellus*) *aquaticus cyclobranchialis* Sket, 1965	
	Asellus (*Asellus*) *aquaticus irregularis* Sket, 1965	
	Asellus (*Asellus*) *aquaticus longicornis* Sket, 1965	
	Proasellus anophtalmus bosnicus (Sket, 1965)	
	Proasellus coxalis nanus Sket, 1990	
	Proasellus deminutus (Sket, 1959)	
	Proasellus intermedius intermedius (Sket, 1965)	
	Proasellus orientalis (Sket, 1965)	

Classification	Taxon described	Category
Asellidae	*Proasellus parvulus* (Sket, 1960)	
	Proasellus slavus histriae (Sket, 1963)	
	Proasellus slavus serbiae (Sket, 1963)	
	Proasellus slavus styriacus (Sket, 1963)	
	Proasellus slavus zeii (Sket, 1963)	
	Proasellus slovenicus (Sket, 1957)	
	Proasellus vulgaris (Sket, 1965)	
	Remasellus Bowman & Sket, 1985	
Atlantasellidae	***Atlantasellidae* Sket, 1979**	***F***
	***Atlantasellus* Sket, 1979**	***G***
	Atlantasellus cavernicolus Sket, 1979	
Brasileirinidae	***Brasileirinidae* Prevorčnik, Ferreira & Sket, 2012**	***F***
	***Brasileirinho* Prevorčnik, Ferreira & Sket, 2012**	***G***
	Brasileirinho cavaticus Prevorčnik, Ferreira & Sket, 2012	
Cirolanidae	*Sphaeromides virei mediodalmatina* Sket, 1964	
	Sphaeromides virei montenigrina Sket, 1957	
	Turcolana lepturoides Prevorčnik, Konec & Sket, 2016	
Lepidocharontidae	*Microcharon luciae* Sket, 1990	
Protojaniridae	*Anneckella srilankae rectecopulans* Sket, 1982	
	Anneckella srilankae srilankae Sket, 1982	
	Enckella lucei major Sket, 1982	
Sphaeromatidae	***Bilistra* Sket & Bruce, 2004**	***G***
	Bilistra cavernicola Sket & Bruce, 2004	
	Bilistra millari Sket & Bruce, 2004	
	Bilistra mollicopulans Sket & Bruce, 2004	
	***Merozoon* Sket, 2012**	***G***
	Merozoon vestigatum Sket, 2012	
	Monolistra (*Microlistra*) *bolei* (Sket, 1960)	
	Monolistra (Microlistra) bolei bolei (Sket, 1960)	
	Monolistra (*Microlistra*) *bolei brevispinosa* Sket, 1982	
	Monolistra (*Microlistra*) *calopyge* Sket, 1982	
	Monolistra (*Microlistra*) *fongi* Prevorčnik, Verovnik, Zagmajster & Sket, 2010	
	Monolistra (*Microlistra*) *jalzici* Prevorčnik, Verovnik, Zagmajster & Sket, 2010	
	Monolistra (*Microlistra*) *pretneri* Sket, 1964	
	Monolistra (*Microlistra*) *pretneri pretneri* Sket, 1964	
	Monolistra (*Microlistra*) *pretneri spinulosa* Sket, 1965	
	Monolistra (*Monolistra*) *coeca intermedia* Sket, 1964	
	Monolistra (*Monolistra*) *monstruosa* Sket, 1970	
	Monolistra (*Monolistrella*) Sket, 1964	
	Monolistra (*Monolistrella*) *velkovrhi* Sket, 1960	
	Monolistra (*Pseudomonolistra*) *bosnica* Sket, 1970	
	Monolistra (*Pseudomonolistra*) *hercegovinensis atypica* Sket, 1965	
	Monolistra (*Pseudomonolistra*) *hercegovinensis brevipes* Sket, 1965	
	Monolistra (*Pseudomonolistra*) *radjai* Prevorčnik & Sket, 2007	
	Monolistra (*Typhlosphaeroma*) *bericum hadzii* Sket, 1959	
	Monolistra (*Typhlosphaeroma*) *matjasici* Sket, 1964	
	Monolistra (*Typhlosphaeroma*) *racovitzai conopyge* Sket, 1964	
	Monolistra (*Typhlosphaeroma*) *racovitzai karamani* Sket, 1959	
	Monolistra (*Typhlosphaeroma*) *racovitzai pseudoberica* Sket, 1964	
Stenasellidae	*Magniezia studiosorum* Sket, 1969	
Chordata		
Amphibia:Proteidae	*Proteus anguinus parkelj* Sket & Arntzen, 1994	
Teleostei: Nemacheilidae	*Triplophysa longibarbata* (Chen, Yang, Sket & Aljančič, 1998)	

Second, the intricate leech taxonomy was frustrating. As he stated in his interview with Traudl Krapp in Amphipoda Newsletters 40, the unsolved taxonomy of erpobdellid leeches motivated him to integrate molecular methodology into taxonomic expertise. In the mid 1990-ies he initiated Peter Trontelj's study visit to Tübingen, where he acquired training in molecular systematics. Upon return to Ljubljana, Peter established a molecular laboratory within the Boris' research team. Molecular phylogenies offered a new perspective on leech taxonomy. Some lineages, such as Erpobdellidae, emerged as well-supported monophyla (Trontelj et al. 1996). In a similar line, fine scale analyses confirmed species status of morphological forms within the *Glossiphonia complanata* species complex (Verovnik et al. 1999). More commonly, however, molecular phylogenetic structure deviated from traditionally accepted groupings and challenged higher taxa such as Rhynchobdellidae (Trontelj et al. 1999), or unveiled inadequate taxonomy in erpobdellid genera *Dina* and *Trocheta* (Trontelj and Sket 2000).

Crustacea: Decapoda

The cave shrimps of the genus *Troglocaris* are remarkable and common animals of the subterranean waters of the Dinaric Karst. Boris admired cave shrimps since he was a boy, but his contributions to knowledge about cave shrimps were mostly revisionary (but see Jugovic et al. 2019), confronting morphological and molecular variation of subterranean freshwater atyid shrimps in Europe. The molecular phylogeny of cave shrimps and surface atyids in Europe revealed unexpected phylogenetic relationships within the group, identified potential new species of cave shrimps and highlighted the necessity for taxonomic reassessment of the group (Zakšek et al. 2007). A comprehensive phylogeographic study of the species with holodinaric distribution pattern, i.e., *Troglocaris anopththalmus* species complex, showed that some large-ranged species are genetically deeply structured, possibly comprising several species (Trontelj et al. 2009; Zakšek et al. 2009). Under Boris' mentorship, PhD student Jure Jugovic utilized these phylogenetic insights to demonstrate that much of the morphological variation observed in cave shrimps is sex and age specific, that size alone is not indicative of an individual's age, and that adults represent the taxonomically most distinct stage in *Troglocaris* (Jugovic et al. 2010a). Furthermore, their research underscored that rostrum length, a traditionally important taxonomic character, is influenced by the presence of predatory olms, rendering it unreliable for species diagnosis (Jugovic et al. 2010b).

Using molecular phylogenies and reliable taxonomic characters, Boris and co-authors redefined the subgeneric structure of the genus *Troglocaris* (Sket and Zakšek 2009), described new species of cave shrimps (Sket and Zakšek 2009; Jugovic et al. 2011), as well as disentangled the species structure of the *Troglocaris anophthalmus* species complex (Jugovic et al. 2012).

Crustacea: Isopoda

Many of Boris' contributions to isopod taxonomy can be considered as footprints of his cave explorations around the globe, including Africa (Sket 1969), Bermuda (Sket

1979a), Sri Lanka (Sket 1982a), Mediterranean region (Sket 1990b), Philippines (Botosaneanu and Sket 1999), New Zealand (Sket and Bruce 2004) and Brazil (Prevorčnik et al. 2012). These expeditions yielded unexpected finds that contributed to global taxonomy of Asellota, Cymothida, Sphaeromatida and Calabozoa, with descriptions of new species, genera and families (e.g. (Sket 1985a).

Nevertheless, Boris' systematic isopod research primarily focused on groups inhabiting the Dinaric Karst and broader Mediterranean Region. He authored the very first revisions of the taxonomy and distribution of surface and subterranean populations of *Asellus aquaticus* (Sket 1963, 1965b, 1994a), an isopod that subsequently became a model for studying cave colonization, morphological differentiation and speciation. Other contributions addressed taxonomic status and occurrence of cirolanids and sphaeromatids in a broader Mediterranean region. Boris's contributions to the knowledge of the relatively few cirolanids of the region were mostly faunistic (Sket 1964a; Delić and Sket 2015), while his taxonomic endeavors focused on partial revisions of generic structures, renaming, and the description of new genera (Prevorčnik et al. 2016; Sket and Baratti 2021).

By contrast, the subterranean sphaeromatids are a common and speciose group, distributed along Dinaric Karst, southern slopes of Alps and Tyrrhenian coast on Apennine Peninsula. Boris laid foundations of taxonomy and biology of subterranean sphaeromatids of the genus *Monolistra* from the Dinaric region, dividing it into subgenera based on sexual size dimorphism, defensive spine structures and degree of reduction of the uropods (Sket 1964b, 1965a, 1982b, 1986e; Prevorčnik and Sket 2007; Prevorčnik et al. 2010).

The marine origin of subterranean sphaeromatids intrigued him deeply. Decades ago, he collected a posterior half of an unknown sphaeromatid in the anchihaline cave Šipun near the town of Cavtat (Croatia). This piece of an animal showed a morphology potentially transitional between a marine ancestor and its alleged descendants from subterranean freshwater (Sket 2012). It has become some sort of holy grail, and several lab members had the mission to complement their holidays at the Adriatic coast with a visit to Šipun Cave. Unfortunately, with no luck yet, so Boris passed away while the riddle still unsolved.

Although terrestrial isopods are common in caves, Boris never looked into their diversity. However, he did provide support for studies conducted by other authors on terrestrial isopods. Nonetheless, he compiled a checklist of this group (Sket 1986d).

Crustacea: Amphipoda

Amphipods were a particular passion of Boris (Fig. 3). He served in international consortia dedicated to compiling global amphipod checklists (Väinölä et al. 2008; Horton et al. 2023). A minor part of his taxonomic works can be associated with his expeditions, resulting in species descriptions from the families Bogidiellidae from China and Ecuador (Sket 1985c; Karaman and Sket 1990a); Sebidae from SE Asia (Jaume et al. 2009), Melitidae from Iran and Philippines (Sawicki et al. 2005; Krapp-Schickel and Sket 2015) and Pseudoniphargidae from Bermuda (Stock et al. 1987).

Figure 3. Left: The parkelj, or Krampus, from Boris' childhood memories, after which he named the black olm, *Proteus anguinus parkelj*. Right: Boris' favourite amphipods, *Niphargus balcanicus* (upper), and *Jugogammarus kusceri* (bottom). (Photo: Boris Sket archive).

More systematic research was devoted to two families, the predominantly subterranean Niphargidae and predominantly epigean Gammaridae s. lat. The genus *Niphargus* was the one that attracted his attention and some of his earliest papers are reports on Niphargidae (Sket 1956, 1958, 1960). His research of niphargid biology revolved around a few key topics. He described several species, subspecies and genera (Sket 1974, 1981b; Karaman and Sket 1989, 1990b; Sket 1990a; Sket and Karaman 1990; Sket 1999b; Fišer et al. 2006a, 2007a; Sket and Karaman 2018; Karaman and Sket 2019). To contribute towards the complex taxonomy of the family, he trained his PhD student, Cene Fišer. Boris had an excellent overview of the niphargid morphological variation, including changes of morphology during development (Sket 1974; Fišer et al. 2008b). He dearly hoped that the high number of species within genus *Niphargus* could be classified into a few phylogenetically supported subgenera. To this end, he conducted several revisions with in-depth discussions on individual characters (Sket 1971, 1972), followed by a few partial cladistic revisions (Sket and Notenboom 1993; Fišer et al. 2006b, 2010) and pioneering attempts of web-based morphological taxonomy that were ahead of their time (Fišer et al. 2009b, 2009a). The first molecular phylogenies, however, revealed a repeated evolution of multiple convergences, with no reliable characters that could satisfactorily diagnose subgenera (Fišer et al. 2008a; Trontelj et al. 2009).

His interest in Gammaridae s. lat. manifested relatively late and was closely tied to his passion for the fauna of ancient freshwater lakes. His vivid interest for ancient lakes resulted in the descriptions of species from the gammaridean family Anisogammaridae from the Chinese lake Fuxian Hu (Sket 2000; Sket and Fišer 2009). An important breakthrough in gammaridean research was a global phylogenetic analysis of the Gammaridae s. lat., conducted in collaboration with Chinese researches. In this study was shown that gammarids colonized freshwater multiple times and subsequently diversified in it (Hou et al. 2011), yet diversification patterns of different lineages varied between evolutionary stasis and rapid diversifications corresponding to adaptive radiations (Hou et al. 2014). These influential studies fully exposed the extent of the taxonomic complexity of Gammaridae s. lat., which encompass several morphological distinct yet phylogenetically non-justified families and genera (Hou and Sket 2015; Sket and Hou 2018b). The phylogenetic framework prompted several attempts to revise the taxonomic structure of the family (Hou and Sket 2015; Sket and Hou 2018a), as well as discussions on taxonomic status of several species complexes (Mamos et al. 2014; Sidorov et al. 2018; Sket et al. 2019; Hou et al. 2022).

Other taxa

The Dinaric stygofauna is renowned for its exotics, which include suspension feeders like sponges, subterranean hydroids, clams, tube worms, and notably, the olm, the only European subterranean amphibian. Boris made significant contributions to the taxonomy and overall understanding of all these species.

Boris described the first troglobitic sponge, *Eunapius subterraneanus*, which was later primarily studied by his teaching assistant, Milan Velikonja. Together, they compiled an overview of the distribution and taxonomic status of both obligate and non-obligate subterranean sponges (Sket and Velikonja 1986).

The only Dinaric subterranean hydrozoan was discovered accidentally in preserved samples. This weird animal was initially noticed by Boris' technical assistant, France Velkovrh. When he reported his finding to Boris and their superior, Prof. Janez Matjašič, his report was met with disbelief. Subsequent examinations confirmed the presence of the subterranean species in the Rak Channel of the Planinska jama (Postojna Planina cave system). In recognition of France Velkovrh's contribution, the enigmatic cnidarian was named as *Velkovrhia enigmatica* (Matjašič and Sket 1971).

Although Boris was not the primary describer, his work played a crucial role in the recognition of the unique subterranean clam *Congeria kusceri*. The species was described in 1962 by Professor Jože Bole. At that time, malacologists considered *Congeria* to be an extinct genus, known only from diverse and widespread fossil records. The original description of the species was in Slovene, which led to it being overlooked internationally. The collaboration with Brian Morton resulted in a systematic revision of morphology and extensive review of the biology of this living fossil (Morton et al. 1998). Later on, Boris challenged the validity of its classification within the genus *Congeria*, and proposed it be reclassified under the extant genus *Mytilopsis* (Sket 2011). This proposal, however, received little attention and is not consistent with molecular phylogeny.

Boris made significant contributions to our understanding of the natural history of the olm through several influential papers. Most notably, he described the non-troglomorphic form discovered in the late 1980s in Bela Krajina, naming it *Proteus anguinus parkelj* (Sket and Arntzen 1994; Arntzen and Sket 1996, 1997). The discovery of the black proteus was a major surprise, and Boris openly admitted that he "envied Andrej Mihevc who actually caught the first specimen." The description was augmented by allozyme polymorphism data, which was the standard molecular taxonomic tool of that time. Based on distributional evidence, Boris hypothesized that i) the olm was a recent, post-Pleistocene colonizer of caves, ii) which colonized caves in several colonization events, from iii) the ancestral surface populations that were already genetically differentiated, and iv) that subterranean populations convergently evolved a similar morphological phenotype (Sket 1997).

Finally, his curiosity extended beyond the metazoan life: he encouraged the first explorations of the microbial composition of the "cave gold" in Slovenia (Megušar and Sket 1977). Recognizing the importance of biofilm, he kept eyes open to pursue this topic. His patience was paid-off decades later, with two studies. The first follow up of the early explorations of cave gold revealed a completely unknown bacterial flora in the Slovenian cave Pajsarca (Pašić et al. 2010). The second study meticulously explored the physical and biological structure of a sprout-like biofilm from Vjetrenica, uncovering a diverse microbial flora and the complex physical structure of cortex and medulla of these sprouts (Kostanjšek et al. 2013).

The origin and evolution of subterranean fauna

The question of the origin of subterranean organisms was a recurring theme in Boris' discussions, albeit in various contexts. He viewed evolution as a fundamental aspect of the scientific work in speleobiology. His primary questions regarding most speleobiological phenomena were "how or why did it develop, why did it happen—to be different from the epigean?"

The origin of subterranean organisms was a topic of lively debate in the 1970s and the 1980s. Researchers recognized the relatedness between subterranean aquatic organisms and both freshwater and marine faunas, suggesting its dual origin. Boris hypothesized that Dinaric subterranean aquatic fauna derived i) directly from marine ancestors, ii) directly from the freshwater ancestors, and iii) from marine ancestors via transitionary surface freshwater phase. He inferred the epigean ancestry of Dinaric subterranean fauna based on his observations of global species distributions. The olm as an amphibian, clearly derived from freshwater species (Sket 1997). Likewise, the origin of subterranean water lice (*Asellus aquaticus*) or *Synurella ambulans* in presence of surface populations was not in question. The cave tube worm (*Marifugia cavatica*) is a marine element, that presumably colonized freshwater through anchialine caves or submerged springs. This hypothesis was consistent with molecular phylogeny indicating that the close relatives of the cave tube worm are *Ficopomatus* living also in the Adriatic Sea. Importantly, *Ficopomatus* species live in a wide range of salinities, from fully marine to

brackish waters (Kupriyanova et al. 2009). An intriguing case presented subterranean sphaeromatid isopods. Sphaeromatidae are predominantly a marine crustacean family. However, Boris noted that the distribution of some species followed the boundaries of paleodrainage basins, leading him to assume that marine species initially colonized surface freshwater, dispersed, and speciated in the surface realm before subsequently colonizing the subterranean realm (Sket 1986b).

Boris argued that subterranean realm was colonized independently on different occasions, refereeing, for example, to morphology and distribution of water lice and olm (Sket 1994a, 1997; Turk et al. 1996). This hypothesis was supported later by molecular phylogenies (Verovnik et al. 2004). He suggested that the varying degrees of morphological similarity between subterranean organisms (in comparison to surface ancestors) resulted from convergent evolution during cave colonization (Sket 1985c, 1997; Turk et al. 1996). Generally, Boris was critical of comparisons between distantly related surface and subterranean species, advocating for model systems comprising closely related species, ideally sister pairs (Simčič and Sket 2019, 2021). To this end, he encouraged research on *Asellus aquaticus*. The efforts of his PhD students and close associates, Simona Prevorčnik, Rudi Verovnik and Peter Trontelj, resulted in Europe-wide phylogeography and extensive morphometrics of water lice providing the evidence that i) Dinaric region acted as a refugium during Pleistocene glaciations, ii) most subterranean populations are genetically completely isolated from adjacent surface populations despite occasional contact, and iii) water lice colonized caves on several occasions relatively recently (Sket 1994a; Verovnik et al. 2003, 2004, 2005). Morphological analyses revealed a rather uniform morphology of surface populations (Prevorčnik et al. 2009), while subterranean populations showed substantial differences from surface ones, including the lack of eyes and pigment. Nevertheless, subterranean populations varied among different caves, suggesting imperfect convergence due to differences among subterranean habitats (Turk et al. 1996; Sket 1997; Prevorčnik et al. 2004).

Boris vividly disagreed with many peers who hypothesized that cave animals no longer evolve. He supported his claims with evidence from various cases, including species living in cave hygropetric environments and *Niphargus* amphipods. Cave hygropetric is a habitat of a permanent weaker or stronger current flowing along the vertical cave rock. Boris became aware of this peculiar subterranean habitat when he noted that some species were regularly found in it (Sket 2004). Despite being unrelated, e.g., beetles and amphipod crustaceans, these species shared characters such as prehensile claws and filter-like mouthparts. This similarity suggests that cave hygropetric is a distinct habitat within the subterranean environment, and some specialized subterranean inhabitants exploit its resources (Sket 2004). A different, yet compatible line of reasoning was used to explain the enormous morphological variation in the amphipod genus *Niphargus*. Boris suggested that *Niphargus* variation in morphology could be associated with ecological differentiation (Sket 1999a). Phylogenetic analyses suggested that much of this variation cannot be attributed to cladogenetic events alone (Fišer et al. 2008a). Many morphologically similar species evolved multiple times (Trontelj et al. 2009). These cases of convergence within entire subterranean clades indicated that the vague term "subterranean environment" comprises replicated subterranean habi-

tats with similar selection pressures, and ongoing evolution within the subterranean realm. The proximal mechanism driving morphological variation could be attributed to heterochrony (Fišer et al. 2008b).

Biodiversity patterns and biogeography

Since his early career, Boris paid attention to data collection and data management. His systematic collection of species distributions in the Western Balkans predated the computer era. The very first records were kept in registers on data-cardboards. These were later digitized in MS Word and MS Excel files. The systematic storage using relational databases began when Maja Zagmajster enrolled in her PhD program, resulting in the development of the SubBioDB database (Zagmajster et al. 2012).

Boris was deeply interested in biogeographical and biodiversity patterns at different scales, asking, for example, where the areas with the highest numbers of troglobionts are, what the general biodiversity patterns are, how much these patterns can be trusted, and which factors shaped them. He advocated that species richness needs to be corrected by the size of a region, and demonstrated that the Dinaric Karst was a global hotspot in subterranean species richness. In studies of biodiversity patterns, he served as both a collaborator and a primary investigator. His biogeographic opus revolved around the distribution of species-rich caves and regions, and biodiversity patterns within the Dinaric Karst.

Species-rich caves and species-rich regions

In 2000, David Culver and Boris Sket published one of the most influential papers in subterranean biology, addressing a straightforward question: how many "species-rich" caves, each counting 20 or more troglobionts are there, and where in the world they are (Culver and Sket 2000). By introducing the arbitrarily defined measure of "richness" or "hotspot," this paper enabled the scaling of any faunistic list and established a comparative framework for studying species richness in individual caves. It marked a milestone in the exploration of subterranean hotspots and patterns of subterranean biodiversity. Noteworthy, the paper already indicated that most of species-rich caves are located outside the tropics, at mid-latitudes.

Data from Slovenian caves facilitated further pioneering spatial studies led by David Culver. An analysis of Slovenian subterranean species richness showed that the spatial position of hotspots was stable and could be predicted from the position of species-rich caves, that the species composition of the region is far from complete (Culver et al. 2004a) and that the length of cave passages, their altitude and depth may predict terrestrial species richness (Culver et al. 2004b). These regional studies grounded considerations of the first global analysis that eventually resulted in another influential hypothesis of "mid-latitude ridge of high subterranean species richness", stating that the regions with the highest numbers of species were aligned along mid-latitudes, presumably reflecting the availability of habitat and high productivity on a surface (Culver et al. 2006).

Dinaric Karst as a global hotspot in subterranean biodiversity

Spatial representations of the collected data on the map of the Western Balkans brought Boris to three main findings.

First, the patterns of subterranean terrestrial and aquatic fauna differ. While terrestrial species richness peaks in the NW and SE, aquatic species richness is highest in the NW (Sket 1994b; Sket et al. 2004a). Detailed analyses decades later corroborated this observation, even after taking into account the spatial extent of analysis and sampling bias (Zagmajster et al. 2008, 2010; Bregović et al. 2019).

Second, Boris recognized that subterranean taxa in the Western Balkans can be classified into five major biogeographic groups (Sket 1994b). Some taxa, such as the olm, cave shrimp and tube worm had holodinaric distribution, spanning from the northwest margins of the Dinaric Karst to the political border between Herzegovina and Montenegro for *Proteus* and *Marifugia*, and even beyond for *Troglocaris*. This distribution was subsequently confirmed in later studies, although recent research acknowledges that these patterns pertain to the genus or species complex level (Sket 1997; Fišer et al. 2007a; Zakšek et al. 2009). Other groups of taxa were found to inhabit smaller areas within the Dinaric Karst, displaying a merodinaric distribution, which encompassed the northwest, southeast, epi-, and paralittoral compartments of the Dinaric Karst. Apart from these, Dinaric Karst inhabit also transdinaric species, which extend their distributional ranges beyond the Dinaric Karst, either in southern Europe or in the southeastern Mediterranean region (Sket 1994b).

Third, Boris hypothesized that distribution patterns are primarily associated with geological history, whereas recent ecological conditions and dispersal play only minor roles. He observed that some species distributions follow paleo-drainages rather than recent ones (Sket 1986b, 2002). He assumed that distributional patterns of the subterranean species were often shaped already on the surface, prior the surface ancestor in multiple colonization events evolved into subterranean descendant (Sket 1994b). By comparing distributional patterns of surface and subterranean relatives, he suggested that distribution of Dinaric subterranean species should be associated with disjunct karstification centers of the Dinaric Karst, the extent of the Pannonian Sea and drying up of the Paratethys, the Messinian Crisis, and Pleistocene glaciations (Sket 1981a, 1988, 1994b). Calibrated molecular phylogenies subsequently provided additional evidence that much of the Dinaric fauna pre-dated the Pleistocene (Trontelj et al. 2007).

On a local scale, Boris acknowledged the significance of ecological dynamics, which emerged as an interplay between interspecific competition and ecological specialization. His analyses of fauna associated with anchialine caves (Sket 1977, 1986a) and thermal water (Sket and Velkovrh 1981a) provided indirect evidence that physical and chemical properties of water could deterministically shape species distribution. In many papers he assumed a covariation between the degree of species ecological specialization and species competitive strength, resulting in outcompeting weaker generalists bymore specialized species (Sket 1981a, 1986a). He never doubted the role of interspecific relationships and used it as a *post hoc* explanation for distribution of many species (Sket 1986a), as a

mechanism for maintaining allopatric distributions (Sket 1994b) and as a possible driver of the colonization of the subterranean realm (Sket 1981a, 2002). His later research showed that the outcome of the interspecific relationship between surface and subterranean species might be less predictable than previously thought (Fišer et al. 2007b).

Ecology

The properties of subterranean environment such as darkness, oligotrophy and stable conditions have rendered ecology an inevitable part of subterranean biology. Understanding the diversity of ecological factors within the subterranean realm was probably pivotal for Boris' views on imperfect convergent evolution (previous section), and also shaped his opinion on threats to subterranean ecosystem. Boris examined the interaction between organism and its environment from two aspects.

The old question, how to treat species found in a cave, Boris addressed theoretically (Sket 2008b). In his review, he was seeking for the simplest compromise among existing classifications of cave organisms, and proposed criteria for their delimitation based on the ecology of species' life cycles. An essential takeaway from his own observations was the necessity for rigorous testing to determine the ecological status of a species, emphasizing that conclusions should not be based solely on superficial impressions. He highlighted that troglobionts may not necessarily exhibit troglomorphism, and conversely, surface-dwelling animals can lack eyes.

In addition, Boris studied the variation of ecological conditions within the subterranean ecosystems in conjunction with community composition. He significantly advanced our understanding of sinking streams, anchihaline caves, fissure systems, and cave hygropetric. Sinking streams were explored in Postojna-Planina cave system between 1965–76 (Sket 1970, 1979b; Sket and Velkovrh 1981b). Sket and collaborators regularly monitored 16 sampling sites along the Pivka River. They measured annual variation in temperature, oxygen, nitrates and bacterial oxygen consumption, as well as community structure. Apart from the updated checklist of the system and vicinity (Sket 1979b), they showed daily and annual temperature fluctuations, gradually declining in dependence of distance from sink and water volume, i.e. the impact from the surface penetrated deeper into cave system at high water level and strong currents. Moreover, they showed that water during its flow through the cave gets oxygenated, whereas nitrogen wastes remain intact (Sket 1970). These studies were a basis for a Slovenian-Brazilian bilateral project 50 years later, with the aim of geographically broadening the study system and evaluating the impact of decades of anthropogenic activities on subterranean biota. Boris was the leader of the Slovenian team, the results still pending the final publication.

As a part of his investigation of the Postojna-Planina cave system, Boris paid focused on water drips. In collaboration with Anton Brancelj and Cvetka Žagar, they showed that these waters harbor unique communities, primarily dominated by copepod crustaceans (Sket 1981b; Sket et al. 2004b). The study allowed the discovery of

a point endemic, *Niphargobates orophobata*, found in a single jet of percolating water. Their findings underscored the exceptional nature of the fauna inhabiting percolated water and significantly contributed to our understanding of epikarst and water-filled fissure systems (Sket 1981b).

Boris conducted pioneering research on the ecology of anchihaline caves globally, focusing on several caves along the Adriatic coast (Sket 1986a, 1996b) (Fig. 4). Water chemistry measurements clearly showed three layers of water: bottom marine, uppermost freshwater and an intermediate, thin layer of halocline with depleted oxygen and rapid transition from poly- to oligohaline conditions. Each of these layers comprised unique ecological habitat, each supporting its own fauna. Using comparative data, Boris elegantly demonstrated that species vertical distribution within the anchihaline water column reflected the interplay between species needs for abiotic environment and interspecific interactions. For example, the amphipod *Niphargus hebereri*, predominantly found in fresh- and only rarely in brackish water, preferred freshwater layer despite its tolerance for mesohaline water; its distribution mostly reflected species' habitat choice. By contrast, the thermosbenacean *Monodella halophila* was found in a presumably predation-free zone within the halocline layer, although it lives in freshwater. The distribution of this species was indicative of its generalistic nature and weak competitiveness (Sket 1986a, 1996b).

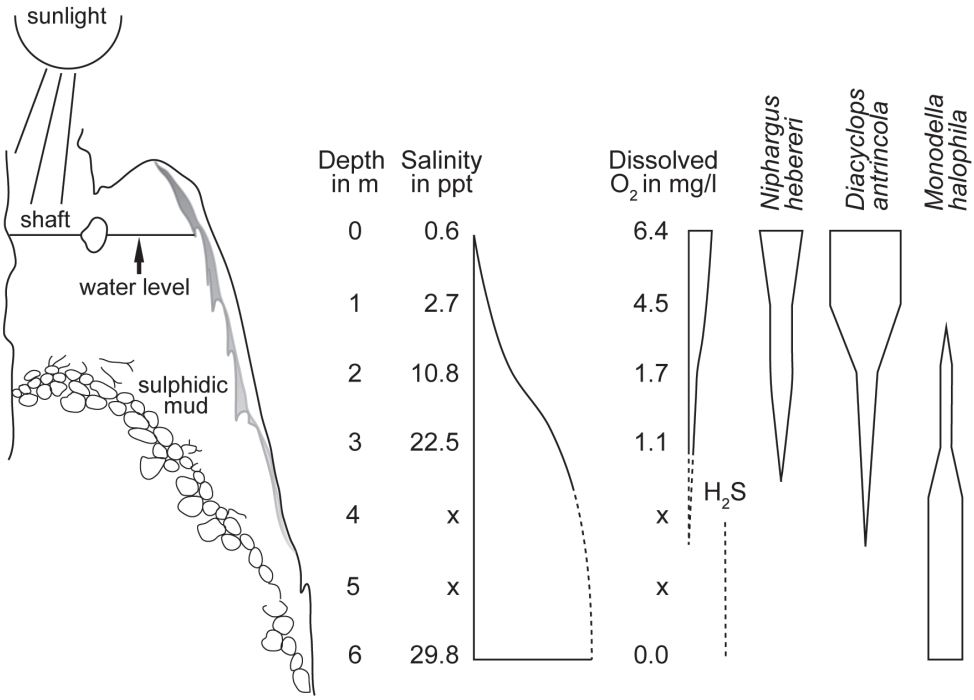

Figure 4. Ecological stratification of water column in anchialine caves. Boris was one the first who studied the vertical stratification of abiotic factors and with it associated community structure. After Sket 1896.

Conservation biology

Boris advocated that subterranean fauna comprises an important part of global and Slovenian natural heritage. His analyses of Slovenian subterranean fauna revealed that the proportion of subterranean species in Slovenia surpassed that found on a global scale (Sket 1999a, 1999c). An important part of his research was devoted to recognition of threats and processes that could aid in the protection of subterranean natural heritage.

He early realized that cave fauna is threatened by the anthropogenic disturbance originating at the surface. One of his early notifications was that of organic pollution in the subterranean flow of the Pivka River in the Postojna-Planina Cave system. This pollution led to an influx of immigrants from the surface and the subsequent disappearance of specialized subterranean species. Boris presumed that eutrophication weakened surface-subterranean barrier resulting in altered community structure and increased interspecific competition pressure on subterranean species (Sket 1970).

Most of subterranean species are endemic (Bole et al. 1993). This view was further rectified by systematic analyses conducted within the European project PASCALIS (Deharveng et al. 2009). Many species live only in small areas, and subterranean communities are characterized by a high beta diversity (Malard et al. 2009), leading to high global (gamma) diversity. Species with large distribution ranges - i.e., larger than 200 km - are often taxonomic artifacts (Trontelj et al. 2009). Boris argued that endemicity, in conjunction with K-strategies, makes subterranean fauna vulnerable (Sket 1999a, 1999c).

To actively contribute to the protection of subterranean fauna, Boris undertook several initiatives. He prepared a series of checklists (Sket 1986f; Sket et al. 1991, 2004a) and conducted assessments of the endangerment status for various species in Slovenia, including leeches (Sket 1992c, 1996a), crustaceans (Sket 1992d; Sket and Brancelj 1992), amphibians (Sket 1992e), and the other species from groundwater (Sket 1992f). Together with David Culver, Boris provided recommendations for the monitoring of caves, advocating for the standardization of sampling effort using fixed time-person units, as well as the use of baiting of terrestrial pitfall traps and aquatic traps, as well as the potential utilization of the capture-mark-recapture method, with a caution note that an increase of population size may indicate eutrophication (Culver and Sket 2002). He also developed criteria and provided a list of caves as habitat type "caves not open to the public" of the Annex II of the Habitat's Directive, that are part of Natura 2000 network in Slovenia.

Several efforts were made to safeguard species-rich caves (Sket 1992a) and/or regions (Michel et al. 2009). Boris had ambitious plans that aimed to establish a network of species-rich regions along the Dinaric Karst, ultimately seeking UNESCO protection. Unfortunately, these efforts were in vain. Boris firmly believed that protecting Slovenian rich-natural heritage is our moral imperative. The message he frequently reiterated was "There is no reason to be proud of our natural heritage, as long the Pivka River draining through the global subterranean hotspot of Postojna-Planinska Cave system, remains polluted".

Figure 5. Field work. (Photo: Boris Sket archive).

The outreach

Boris stood out as one of the most prominent and influential zoologists in Slovenia. As a professor at the University of Ljubljana, he played a pivotal role in shaping the education of numerous generations of biology students and teachers. While teaching courses such as Invertebrate Zoology and Evolution, he also introduced the subject of "Subterranean Biology" into the biology curriculum. Under his mentorship, ten PhD students, five MSc students, and 25 graduate students successfully completed their studies. Many of these individuals have become respected zoologists in various research fields. Among these, we must mention Milan Velikonja, who studied subterranean sponges, Anton Brancelj, who established the model of epikarst and became one of the world-leading taxonomists for microcrustaceans, Tone Novak, who studied cave fauna outside Dinaric Karst with an emphasis on opilionid taxonomy and physiological adaptations, and the research team SubBioLab.

Beyond his teaching and research endeavors, he also paid attention to broader audience interested in natural sciences. He regularly contributed to the popular science magazine "Proteus," sharing his insights and knowledge with a wider readership. Furthermore, in the 1970s, he edited a series of identification keys for various groups of animals, making valuable information accessible to enthusiasts and researchers alike. Together with Meta Povž, he co-authored a comprehensive book on Slovenian fishes in 1990. This impressive

volume detailed the Slovenian fish fauna, providing descriptions and insights into the biology of various species. Moreover, Boris was a writer and co-editor of the expansive monograph "Živalstvo Slovenije" (The Fauna of Slovenia). This exhaustive volume presented Slovenian fauna in an accessible and comprehensive manner, covering anatomy, ecology, and diversity, catering to students and naturalists alike. Lastly, he authored a high school textbook on Evolution, further contributing to science education at various levels.

Boris influence extended beyond the biological and naturalist communities. Through his writings in daily newspapers, he persistently advocated for the protection of our natural heritage, with a voice of a man who eyewitnessed the transformation of society and environmental degradation. He was one of the giants, whose shoulders allow us seeing further.

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Supplementary material 1

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