Geographically structured genetic diversity in the cave beetle *Darlingtonia kentuckensis* Valentine, 1952 (Coleoptera, Carabidae, Trechini, Trechina)

Olivia F. Boyd¹², T. Keith Philips¹, Jarrett R. Johnson¹, Jedidiah J. Nixon¹

¹ Department of Biology, Western Kentucky University, Bowling Green, KY 42101 USA ² Department of Integrative Biology, Oregon State University, Corvallis, OR 97331 USA

Corresponding author: T. Keith Philips (Keith.Philips@wku.edu)

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Abstract
Cave beetles of the eastern USA are one of many poorly studied groups of insects and nearly all previous work delimiting species is based solely on morphology. This study assesses genetic diversity in the monotypic cave carabid beetle genus *Darlingtonia* Valentine 1952, to test the relationship between putative geographical barriers to subterranean dispersal and the boundaries of genetically distinct groups. Approximately 400bp of the mitochondrial cytochrome oxidase I (COI) gene was sequenced from up to four individuals from each of 27 populations, sampled from caves along the escarpments of the Mississippian and Cumberland plateaus in eastern Kentucky, USA. The 81 individuals sequenced yielded 28 unique haplotypes. Hierarchical analyses of molecular variance (AMOVA) within and among geographically defined groups tested two *a priori* hypotheses of structure based on major and minor river drainages, as well as genetic distance clusters defined *a posteriori* from an unrooted analysis. High genetic differentiation (*F*<sub>ST</sub>) between populations was found across analyses. The influence of isolation by distance could potentially account for much but not all of the variation found among geographically defined groups at both levels. High variability among the three northernmost genetic clusters (*F*<sub>CT</sub>), low variability among populations within clusters (*F*<sub>SC</sub>), and low within-cluster Mantel correlations indicate the importance of unidentified likely intra-karst barriers to gene flow separating closely grouped cave populations. Overall phylogeographic patterns are consistent with previous evidence of population isolation among cave systems in the region, revealing geographically structured cryptic diversity in *Darlingtonia* over its distribution. The landscape features considered *a priori* in this study were not predictive of the genetic breaks among the three northern clusters, which are genetically distinct despite their close geographic proximity.

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Keywords
mitochondrial DNA, Mississippian Plateau, Pennyroyal, phylogeography, Southern Appalachians, troglobites, troglobionts

Introduction

Variation within a species is usually not random, but structured in some way and typically forms a metapopulation with various levels of deviation from panmixia (Hanski 1999). Landscape features that correlate with intraspecific variation may represent boundaries reducing gene flow among discrete groups of populations. Alternatively, differences between populations of a species may increase linearly with physical distance, especially for less vagile organisms (e.g., Lee and Mitchell-Olds 2011, Goudarzi et al. 2019). The limestone karst regions of the Eastern United States support a remarkable diversity of cave-specialized animals (Barr 1985, Peck 1998, Hobbs 2012, and see White et al. 2019). Troglobionts, i.e., obligate and permanent cave inhabitants, can be predicted to demonstrate high levels of population genetic structure owing to a lack of gene flow between caves. Even long-term population isolation, however, may not yield diagnosable morphological differentiation due to phenotypic convergence in similar cave environments (Wiens et al. 2003, Derkarabetian et al. 2010, Hedin and Thomas 2010). Therefore, many troglobiotic taxa may harbor cryptic variation (Niemiller et al. 2012), and the biodiversity of cave-dwelling organisms may currently be underestimated.

Patterns of gene flow among caves in karst areas vary mostly in accordance with the geographical distribution of subterranean limestone (e.g., Caccone 1985, Katz et al. 2018). In limestone-rich parts of the Eastern United States (Fig. 1) where karst exposure is patchy, structurally fragmented, and discontinuous, caves are generally smaller and more isolated from one another (e.g., Currens 2002, Christman et al. 2005). One such region is the Appalachian Valley (AV), located primarily in eastern Tennessee and Virginia, which supports a high diversity of endemic cave beetles and other troglobites per unit area, many of which are limited in range to one or a few caves (Barr 1967, 1981, 1985, Christman et al. 2005, Niemiller and Zigler 2013). Conversely, troglobiotic invertebrates that inhabit large and highly interconnected cave systems which have permeated the large and uninterrupted exposures of limestone in the Mississippian Plateau (MP) region have comparatively broader ranges and less predictable distributional boundaries (e.g., Barr 1979). Species numbers and abundances differ among cave communities in the interior low (“Mississippian”) plateau (referred to below as “MP”) and Appalachians (Appalachian valley and ridge, referred to below as “AV”) regions; MP cave systems support larger and richer communities of troglobionts compared with those in the AV to the east (Barr and Holsinger 1985). With fewer endemics per unit area, cave species in the MP have been suggested as more likely to occur in sympatry than those inhabiting AV caves (Barr 1967, 1985, 2004). More recently though, Christman et al. (2016) presented contrasting evidence that despite the greater dissection of karst in the AV, cave species actually have lower rates of endemicity in the AV than in the MP.
The cave-rich limestone of the MP is bisected by the Cumberland Saddle, a low point in the Cincinnati Arch formation, which separates the MP into two regions: the MP-I to the west and the MP-II to the east (Fig. 1). Within both bands of the MP, cave interconnectivity has helped establish and maintain diversity by facilitating subterranean dispersal, leading to extensive range overlap and sympatry of species that were previously isolated, and linking populations together through gene flow which likely has reduced stochastic extinction events (Barr 1985, Barr and Holsinger 1985).

Isolating barriers between cave systems restrict gene flow and promote divergence among populations of cave organisms, effectively dividing parts of cave systems into subterranean islands (Culver 1970). Major waterways like the Cumberland and Ohio rivers serve as important fluvial barriers to dispersal of terrestrial troglobionts (Barr and Holsinger 1985, Barr 1985) and even some stygobionts (Niemiller et al. 2013), but smaller streams and rivers may actually promote their dispersal; Barr (1985) compared the “meander frequencies” of rivers dividing the distributions of cave beetle species, finding support for his hypothesis that the more turns a river takes over a given distance, the more often beetles washed out of caves will survive to encounter limestone outcrop karst refugia leading to an increase in distribution range via colonization of new cave systems.

**Figure 1.** (Adapted from Barr 1985, Figure 3) Map showing the major geologic features important for cave development in the southeastern United States: MP-I and MP-II (green) are western and eastern bands of the Mississippian Plateau. Dots indicate collecting records (see Figure 3).
Study species

*Darlingtonaea* Valentine, 1952 is a monotypic genus of cave carabid beetle found in a narrow distributional band from north-central Tennessee (known from a single cave near the Kentucky border) extending northeastward into east-central Kentucky (mainly the northern part of “MP-II” in Fig. 1 and see Fig. 3). Like many of the other cave-specialized carabids of the subtribe Trechina, *Darlingtonaea* are true troglobionts, with adaptations for subterranean life: they lack eyes and wings, possess enlarged mouth-parts, lengthened appendages, and specialized sensory setae, and are depigmented compared with their epigean relatives (Fig. 2). *Darlingtonaea kentuckensis* Valentine is usually abundant in caves within its range compared to many species of closely related *Pseudanophthalmus* (Valentine 1952). Molecular phylogenetic evidence from a 2012 study including representatives of all five eastern North American cave genera shows the genus shares common ancestry with a lineage of *Pseudanophthalmus* and is essentially derived from within the latter (Philips and Valkanas, unpublished). The close relationship of those genera together with *Ameroduvalius* Valentine, *Nelsonites* Valentine, and *Neaphaenops* Jeannel within the *Trechoblemus* series and within the Trechina is also strongly supported by Maddison et al. (2019).

Regarding the origin and diversity of North American cave trechines, most authors have favored some version of a “Pleistocene-effect” model (Holsinger 1988). In contrast, Faille et al. (2015) puts the divergence times between two European trechine *Aphaenops* cave species around 9 my (with a credibility range of 4–17 my). Regardless of age, the proposed evolutionary scenario can be summarized as follows: As climate cycles associated with glacial advance and recession led to fluctuation of surface conditions, ancestral trechines followed cool, moist microhabitats from the deep soil which was abundant during glacial maxima to subterranean or montane refugia during warmer, drier glacial minima (Barr 1969, 1971, 1973, 1985). Periods of isolation in caves during warm intervals were punctuated by periods of introgression during cool intervals until a warm, stable post-Pleistocene climate restricted surface dispersal and promoted subterranean allopatric speciation (and see Jeannel 1948, 1949 for further details on the effects of glaciation).

Other authors have found isolation and divergence in allopatry to be an unsatisfactory model for cave colonization in other taxa, which may be better viewed as a parapatric ecological transition or “adaptive shift” occurring in the presence of gene flow via diversifying selection (Niemiller et al. 2008). Further, surface characteristics of the Earth, such as latitude, percent karst, and landscape rugosity (Topographic Position Index) may have significant effects on the evolution of a cave-adapted fauna (Christman et al. 2016).

It is currently unclear what factors have led to the evolution of any morphological or genetic diversity within *Darlingtonaea kentuckensis*. *Darlingtonaea kentuckensis* has a broader than average distribution compared to most terrestrial Eastern North American troglobionts based on our review (Philips et al. unpublished). Both Valentine (1952) and Barr (1985) noted some morphological diversity among populations of *D. kentuckensis*. For example, Valentine noted subtle differences including a slightly more
convex body form, slightly wider elytra, and more rounded elytral humeral angles (in populations on either side of the Cumberland River), but concluded there was not enough support for subspecific designation. In contrast, the population from Big Salt-peter Cave in Rockcastle County by the Rockcastle River was thought to be distinct enough to warrant the subspecific name *D. k. lexingtoni* Valentine. Morphologically, this taxon diagnosis was based on a slightly paler body color, very slightly narrower pronotum, flatter elytral disc, and claimed differences in the male genitalia that included subtle differences in the apex of the median lobe and one lobe of the internal sac (see Valentine 1952, Plate IV).

Barr (1985) speculated that *D. kentuckensis* includes at least seven subspecies or races isolated by landscape barriers. Kane et al. (1992) sampled ten *D. kentuckensis* populations from across the MP-II for a study of allozyme diversity. Polymorphism in nine of the eleven electrophoretic markers examined combined with the lack of variation within populations and high $F_{ST}$ across loci suggested long-term isolation.

The exceptional species diversity in North American cave trechines (Peck 1998) makes this lineage valuable to understanding the speciation processes in troglobiotic insects and other terrestrial cave organisms. Since populations of *Darlingtonea* occur across a broad geographic range relative to other troglobiotic taxa while belonging to a single morphologically, geographically, and genetically distinct lineage, *D. kentuckensis* is a convenient model for comparing observed patterns of genetic variation against those predicted by a climate-mediated process of cave colonization.
Purpose and hypotheses

If important barriers to dispersal for cave trechines in the MP-II region exist, hierarchical tests of population genetic structure should reveal a general pattern of low diversity within and high diversity among clusters of genetically similar populations. Specific geographic barriers between these genetic clusters that may be responsible for population structure can then be hypothesized and should make geographic sense without being purely attributable to the influence of isolation and genetic divergence by distance. Patterns may also reveal the presence of cryptic species or subspecies.

The Kentucky and upper Cumberland rivers represent the two primary watersheds in the MP-II. Further, the divide between the watersheds of the Kentucky and Rockcastle rivers in northern Jackson County (Barr 1985) and the upper Cumberland River in southern Pulaski County (Barr 1985, Lewis and Lewis 2005) may represent...
two additional major barriers to gene flow. These drainage barriers, along with an additional geological/historical barrier isolating genetically distinct groups of populations in northern and southern Pulaski County (Kane et al. 1992), may effectively divide the sampled range of *Darlingtononea* into four faunal regions (Table 1 and Fig. 4): on the north side (Faunal Region 1) or the south side (Faunal Region 2) of the Kentucky-Rockcastle drainage divide and north (Faunal Region 3) and south (Faunal Region 4) of the Cumberland River. Populations hypothesized by Barr (1985) from a potential fifth faunal region east of the Big South Fork of the Cumberland River were not sampled in this study. “Structure hypothesis I” tested herein predicts that sampled populations fall into four genetically distinct clusters that are geographically consistent with the hypothesis of reduced gene flow among these four major regions subdivided by major river systems.

Caves also fall into smaller, “minor” watersheds (Table 1) that could define components of population genetic structure at a finer resolution, especially if Barr’s (1985) hypothesis about the role of smaller, meandering streams in promoting cave beetle dispersal is valid. Samples from the 27 localities (each from an individual collecting event) in the final data set were assigned to watersheds based on both absolute proximity to second- and third-order streams and qualitative topographic information. Under “structure hypothesis II”, populations are expected to fall into ten genetically distinct clusters, with a pattern of genetic structure that is geographically consistent with reduced gene flow among these ten minor watersheds.

**Methods**

**Collecting**

Collecting localities (Figs 1, 3) were prioritized based upon a technical report compiled by Harker and Barr (1979) for the Kentucky State Nature Preserves Commission that listed caves where the target taxon could be sampled. Inclusion of several additional localities that would have benefited this study was not possible due to cave access restrictions imposed in recent decades by landowners for the prevention of vandalism or by conservation authorities for the protection of the two federally endangered *Myotis* bat species. Appropriate measures were taken as recommended by the most recent national White Nose Syndrome decontamination protocol (v:06.25.2012) to help slow the spread of *Geomycetes destructans* Blehert & Gargas (also known as *Pseudogymnoascus destructans* (Blehert & Gargas) Minnis & D.L. Lindner) the introduced fungal pathogen which has led to recent population declines in many species of North American bats.

Beetle specimens were collected by hand into 95% ethanol and placed at -20 °C for short-term storage within 48 hours of collection. Ethanol was changed after processing (individuals from each locality were sorted by genus and inventoried) and whole specimens from each location were stored together in 95% EtOH at -80 °C. Table 1 summarizes collecting information and group membership relative to each hypothesis.
Table 1. List of *Darlingtonaea* populations included in a study of mitochondrial haplotypes, including population (taxon) reference codes, locality information, collection dates, sample size, faunal region, local watershed and GenBank accession codes. Faunal region 1.

<table>
<thead>
<tr>
<th>Taxon Code</th>
<th>Cave</th>
<th>County</th>
<th>Collection Date</th>
<th>N</th>
<th>Faunal Region</th>
<th>Local Watershed/Code (River Drainage)</th>
<th>GenBank accession number</th>
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<td>BLO</td>
<td>Blowing</td>
<td>Wayne</td>
<td>1-Mar-2014</td>
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<td>4</td>
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<td>Estill</td>
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<td>1</td>
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<td>31-Jul-2014</td>
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<td>4</td>
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<td>John Griffin</td>
<td>Jackson</td>
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<td>4</td>
<td>2</td>
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<td>2</td>
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<td>2</td>
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<td>Rockcastle</td>
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</table>
Geographically structured genetic diversity in the cave beetle Darlingtonea

Figure 4. Distribution of cave collection sites and proportions of haplotypes from 27 populations of Darlingtonea kentuckensis in eastern Kentucky, USA. Circle area corresponds to number of individuals sampled per locality. Different colors indicate different haplotypes; similarity in hue qualitatively indicates sequence similarity. KR: Kentucky River; RR: Rockcastle River; CR: Cumberland River; MVF: Mount Vernon Fault; DD = drainage divide between Kentucky and Rockcastle rivers.

Sequencing

Depending on the number of specimens available, up to four Darlingtonea individuals per cave were sequenced (for a total of 81 specimens) to capture a sample of within-population mitochondrial cytochrome oxidase subunit I (COI) haplotype diversity (Table 1). Gut material, if visible, was removed in order to avoid amplification of foreign DNA from prey or other organisms. Whole specimens were ground inside 1.5 ml tubes using sterile plastic pestles and incubated in a solution of CTL buffer and proteinase K for 18–24 hours at 40 °C. Total genomic DNA was extracted from whole specimens using an E.Z.N.A. Insect DNA kit from Omega Bio-Tek. Nucleic acid
concentration and purity was quantified using a NanoDrop 2000 spectrophotometer. Extractions were stored post-purification at -80 °C for long-term DNA preservation.

An ~850 bp COI target region was amplified from genomic DNA using the primer pair “Pat” and “Jerry” (Simon et al. 1994). Thermal cycling conditions for polymerase chain reaction (PCR) followed those specified by the manufacturer of TaKaRa Ex Taq, which was used for all PCR reactions. Primer annealing temperatures were optimized qualitatively by visualizing PCR products from a temperature gradient on an agarose gel to maximize yield and limit nonspecific binding. A QIAquick Gel Extraction Kit from Qiagen was used to purify most PCR products before sequencing. DNA template samples were prepared for sequencing in the forward direction using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Automated cycle sequencing was performed using an ABI 3130 Genetic Analyzer (Applied Biosystems) at Western Kentucky University.

Sequences were aligned using CLUSTALW (Larkin et al. 2007) using the default settings (gap open cost of 15 and a gap extend cost of 6.66), although no gaps were present. Sequences were then edited manually in Geneious version R7 (http://www.geneious.com, Kearse et al. 2012) according to the following rules: IUPAC ambiguous bases were inserted where peaks in the chromatogram overlapped, making base calls questionable. The ends of sequence reads were trimmed when peaks became indistinct or read quality (%HQ) consistently fell below 20 percent (this was common among reads, especially at the 3’ end, since sequencing was performed in only one direction). Reads were translated and screened for signs of pseudogene amplification, including mid-sequence stop codons and frameshifts. Each offending read was manually inspected: in cases where the correct base was obvious upon inspection of the chromatogram, the sequence was corrected and included; in cases where the correct base was unclear, the sequence was omitted and sequencing was re-attempted for that specimen. All sequences were trimmed evenly to 413 bp to eliminate the considerable variation in sequence length that resulted from quality trimming while maximizing the number of operational taxonomic units (OTUs) included.

Analyses

Partial COI sequences were collapsed into haplotypes using the online tool FaBox (Villesen 2007). Thirty-eight sites (~9%) were variable of 413 total bases in the fragment. Twenty-eight unique COI haplotypes were identified among a total of 81 individuals from 27 caves. Genetic structure among and within sampled populations was evaluated for each geographic partitioning scheme (i.e., hypothesis of structure): (I) across four faunal regions divided by the two major barriers in MP-II, and (II) across 10 minor river drainages to which caves were assigned based on proximity to second- and third-order streams and qualitative topographic information.

Arlequin 3.5 (Excoffier and Lischer 2010) was used to perform hierarchical analyses of molecular variance (AMOVA) for structure hypotheses I and II. Analysis of molecular variance estimates the percentage of genetic variation captured by different pre-
defined hierarchical partitions (e.g. among all regions, among caves within each region, and among all caves). From these statistics, fixation indices (F-statistics) were calculated.

\( F_{ST} \) estimates the degree of differentiation among subpopulations within the total population. The closer \( F_{ST} \) is to 1, the greater the extent of allelic fixation or identity within populations (Holsinger and Weir 2009). \( F_{SC} \) estimates the differentiation among populations within the groups to which they are assigned. The closer \( F_{SC} \) is to 1, the more heterogeneity within groups. \( F_{CT} \) estimates differentiation among those groups of populations. The closer \( F_{CT} \) is to 1, the more divergent the groups are from each other. If strong population genetic structure exists at the group scale being analyzed (i.e., faunal regions), \( F_{CT} \) should be high relative to \( F_{SC} \).

Distance matrices and network connections among COI haplotypes were also calculated in Arlequin. Fixation indices (Weir and Cockerham 1984) were calculated from observed diversity within and among populations at each level of geographic structure, and were compared \( (\alpha = 0.05) \) to a null resampling distribution of variance components generated from 10,000 permutations in Arlequin.

An unrooted split network based on a NeighborNet algorithm was generated in SplitsTree (Huson and Bryant 2006) to identify distinct genetic clusters from all 81 COI sequences without regard to their relationships. These clusters (identified \textit{a posteriori}, in contrast to the \textit{a priori} geographic regions and watersheds in hypotheses I and II) defined the groups for which molecular variance was analyzed for a third structure hypothesis (III).

Network connections among haplotypes were gathered directly from Arlequin output data, and a minimum spanning network of COI haplotypes was constructed using the program HapStar (Teacher and Griffiths 2011). The resulting network was edited in Adobe Illustrator to reflect frequencies of individual haplotypes and their regional associations according to each hypothesis. Mantel tests of association between full matrices and partial submatrices of genetic and geographic distances were performed in R using the package ade4 (Chessel et al. 2004) to detect potential effects of isolation by distance. Mantel tests are commonly performed in studies of population genetics to evaluate the strength of association between genetic and geographic distance (e.g. Diniz-Filho et al. 2013). A high correlation can indicate that some of the population structure observed can be attributed to variation in allele frequencies over geographic distance, which is expected to some degree even in panmictic populations. If a large percentage of genetic variation can be explained by geographic distance, it is difficult to say how much of the observed diversity can be attributed to the particular isolating mechanisms proposed and how much is a consequence of isolation by physical distance (IBD). The population pairwise \( F_{ST} \) matrix was generated in Arlequin, and the geographic distance matrix was generated from a list of decimal degree coordinates using Geographic Distance Matrix Generator v.1.2.3 (Ersts 2015), an online open source tool provided by the Center for Biodiversity and Conservation, American Museum of Natural History.

Due to the nearly identical external morphology in adults, male genitalia was also examined in a specimen from each cave sampled to see if any differences could be found and if so, to see if there was any correlation between groups discovered via the genetic analysis.
Results

Successful PCR amplification was found to be less reliable for older samples (some as old as five years), despite storage at -80 °C in 95% or stronger ethanol. Despite careful optimization of thermal cycling conditions, agarose gel purification of PCR products was found to considerably improve sequence read quality and was performed for most samples included in the final data set.

The distribution of cave collection sites and proportions of haplotypes from 27 populations are shown in Figs 3 and 4 respectively. Frequencies of COI haplotypes and their proportions in each major faunal region (I) or minor watershed (II) are shown in Fig. 5. A minimum spanning network of COI haplotypes is color coded for each structure hypothesis in Fig. 6A–C). A network of the 81 COI sequences (Fig. 6D) reveals five genetically distinct clusters of structure hypothesis III.

The analysis of molecular variance (AMOVA), from which $F$-statistics ($F_{ST}$, $F_{CT}$, and $F_{SC}$) were calculated to describe nucleotide sequence diversity at hierarchical levels, within and among groups from each hypothesis of structure are summarized in Table 2. The first two hypotheses were based upon *a priori* geographical hypotheses: (I) the location of caves

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Variance components</th>
<th>Percentage of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMOVA I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among groups</td>
<td>3</td>
<td>149.425</td>
<td>2.1946 (Va)</td>
<td>56.30</td>
</tr>
<tr>
<td>Among populations within groups</td>
<td>23</td>
<td>108.884</td>
<td>1.44888 (Vb)</td>
<td>37.17</td>
</tr>
<tr>
<td>Within populations</td>
<td>58</td>
<td>14.750</td>
<td>0.25431 (Vc)</td>
<td>6.52</td>
</tr>
<tr>
<td>Total</td>
<td>84</td>
<td>273.059</td>
<td>3.89780</td>
<td>100</td>
</tr>
<tr>
<td>Fixation Indices: I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSC</td>
<td>0.85069</td>
<td>Vb and FSC : P(random &gt; observed) = 0.00000***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FST</td>
<td>0.93476</td>
<td>Vc and FST : P(random &lt; observed) = 0.00000***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FCT</td>
<td>0.56304</td>
<td>Va and FCT : P(random &gt; observed) = 0.00000***</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| AMOVA II            |                    |                |                     |                        |
| Among groups        | 9                  | 196.197        | 2.16311 (Va)        | 60.85                  |
| Among populations within groups | 17            | 62.112         | 1.13762 (Vb)       | 32.00                  |
| Within populations  | 58                 | 14.750         | 0.25431 (Vc)       | 7.15                   |
| Total               | 84                 | 273.059        | 3.55503             | 100                    |
| Fixation Indices: II|                   |                |                     |                        |
| FSC                 | 0.81730            | Vb and FSC : P(random > observed) = 0.00000*** |
| FST                 | 0.92846            | Vc and FST : P(random < observed) = 0.00000*** |
| FCT                 | 0.60846            | Va and FCT : P(random > observed) = 0.00000*** |

| AMOVA III           |                    |                |                     |                        |
| Among groups        | 4                  | 221.073        | 3.27840 (Va)        | 81.93                  |
| Among populations within groups | 22            | 37.236         | 0.46852 (Vb)       | 11.71                  |
| Within populations  | 58                 | 14.750         | 0.25431 (Vc)       | 6.36                   |
| Total               | 84                 | 273.059        | 4.00124             | 100                    |
| Fixation Indices: III|                 |                |                     |                        |
| FSC                 | 0.64818            | Vb and FSC : P(random > observed) = 0.00000*** |
| FST                 | 0.93644            | Vc and FST : P(random < observed) = 0.00000*** |
| FCT                 | 0.81935            | Va and FCT : P(random > observed) = 0.00000*** |
Geographically structured genetic diversity in the cave beetle Darlingtonia

Figure 5. Frequencies of COI haplotypes and their proportions, color coded for each hypothesis of structure; circle area corresponds to number of individuals assigned to each group. Overlaid transparent dots show collecting localities. A Four faunal regions of hypothesis I (fifth region unsampled in this study: see discussion and Barr 1985, Kane et al. 1992) B ten minor watersheds of hypothesis II C five genetic clusters of hypothesis III.

sampled relative to two zoogeographic barriers proposed by Barr (1985) to be biologically important in MP-II, and (II) the ten minor watersheds to which sampled caves were classified based on assumptions about hydrology gathered from topographic maps (see Table 1).

AMOVA for the a posteriori structure hypothesis III, based on five distinct genetic clusters from a neighbor-joining network of COI sequences produced the greatest difference between $F_{CT}$ and $F_{SC}$ among all three analyses. In other words, when nucleotide diversity is partitioned among hierarchical levels, variance in nucleotide diversity is maximized among groups and minimized within groups. The northernmost 15 sampled populations make up three genetic clusters within an approximately ten-kilometer physical radius of one another. In this arrangement, no haplotypes are shared between the three
Figure 6. A–C Minimum spanning networks of COI haplotypes, color-coded for each hypothesis of structure. A Four faunal regions of hypothesis I B ten watersheds of hypothesis II C five genetic clusters of hypothesis III D A split network of 85 COI sequences revealing the five genetically distinct clusters of hypothesis III.

groups, and the clusters contradict both a priori hypotheses about the locations of important major and minor water barriers to gene flow, especially in the northern part of the MP-II. Mantel tests of group submatrices found population pairwise $F_{ST}$ to be independent of geographic distance within each cluster. Among all 15 of these populations, only a maximum of 14% of the observed variation can be explained by geographic distance.

Examination of male genitalia generally showed only slight differences among cave localities examined (Fig. 7). The median lobes were of a consistent shape as were the parameres and internal sac morphology with one notable exception. In specimen 14, the paramere expansion is absent and the internal sac appears to have a different shape.
within the median lobe. This morphology was found only in Hicksey Cave (abbreviated HIC in all Figs) located in the northern part of the distribution. One should note that paramere expansion is more visible in those specimens that have darker cuticle and hence individuals can appear more different than they actually are due to superficial
color differences. No support for a distinct genitalic morphology of *D. kentuckensis lexingtoni* was observed and the three caves sampled with this subspecies (Great Saltpeter, Teamers, and Mullins Spring Caves) are no more distinct than some of the populations from sets of caves or even single caves such as individuals from Pourover Cave.

**Discussion**

$F_{ST}$ measures allelic identity within populations, or among-population variation. Across partitioning schemes, $F_{ST}$ values close to one indicate that individuals within populations are more similar to each other than to individuals in other populations, corroborating the idea that in general, cave populations in this study are isolated from one another. Structure hypotheses I and II were developed based on *a priori* information about the locations of cave collection sites relative to (I) two hypothesized major geographic barriers to gene flow or (II) ten watersheds of higher-order streams. Results of AMOVA for evaluating structure hypotheses I and II indicated that for both hypotheses, the majority of total variation (56–61%) is accounted for by variation among the groups defined under each hypothesis. These results support both structure hypotheses I and II over a null hypothesis of panmixia. Due to the similarity of results for both structure hypotheses I and II and because they are not mutually exclusive, neither can be concluded to better represent geographic structure of genetic diversity among the populations sampled. Hence both the major rivers and even some of the smaller watersheds may be geographic barriers to gene flow. High estimates of $F_{SC}$ relative to $F_{CT}$ (Table 2), as well as shared haplotypes among groups in the northern MP-II indicates that neither hypothesis provides the most optimal scheme for partitioning the observed genetic diversity. The lack of robust support for a partitioning scheme based on small watersheds is not necessarily evidence against the influence of climate cycles on the process of lineage diversification. Many caves do not “belong” to a single watershed, but rather may connect or fall between two or more. This factor, along with the uncertainty surrounding cave connectivity via small passages accessible only by small taxa like these beetles, can make it difficult to truly know the possible connectivity of some caves to one watershed over another. Additionally, it is possible that the shape, size, and the pathway of the watersheds in this area changed throughout the recent Pleistocene and earlier. Hence the separation of populations by hypothesized barriers between caves assigned to different watersheds may have resulted from actual watershed barriers, intra-karst heterogeneity, and or climate cycles at various times that in turn helped drive or prevent cave colonization.

Structure hypothesis III was developed based on the five genetic clusters resulting from a split network. The boundaries for the five population clusters in this hypothesis were determined solely by clustering based on genetic distances among sequences, independently of any *a priori* geographic information. AMOVA statistics for structure hypothesis III (Table 2) indicate that for each hypothesis of structure, among-group variation accounts for a higher percentage of the total variation than within-group
variation. These results support all three hypotheses as better models for structured diversity compared with a null model of panmixia. However, variation among groups (genetic clusters) in hypothesis III accounts for much more of the total variation (82%) than either hypothesis I or II (56% and 61%, respectively). Further, only in structure hypothesis III does diversity among groups (\(F_{CT} = 0.82\)) exceed diversity within groups (\(F_{SC} = 0.65\)). Unlike hypotheses I and II, no haplotypes are shared between the five clusters. Lastly, these five genetic clusters form natural, geographically proximate groupings. Hence the evidence supports hypothesis III as the most representative model for the geographic structure of genetic diversity among sampled populations, and especially for those in the northern MP-II part of the distribution.

If geographic distance is strongly positively correlated with genetic distance, gaps in sampling (rather than specific geographic features acting as barriers to gene flow) could be responsible for at least some of the observed clustering of populations. Results of partial Mantel tests (Table 3) indicate up to 18% of the total observed genetic variation across all 27 populations can be attributed solely to the influence of geographic distance. Across the 15 northern populations (three of the five genetic clusters), IBD could explain up to 14% of the total variation. However, low Mantel correlations for population subsets corresponding to each of these three clusters suggests that the genetic structure observed in this region (Rockcastle, Jackson, and Estill counties) is most likely due to actual barriers to gene flow and not simply isolation by distance.

Barr (1985) recognized that the fragmented geology of Rockcastle County, Kentucky may account for the morphological (and genetic) variability in the region, which is topographically complex and dissected with many rivers and streams. The five clusters (including two completely outside Rockcastle County) could represent distinct lineages important in considering the ecology and evolution of *Darlingtonea*, but divergence times and particular geographic or geologic features consistent with the apparent locations of most putative isolating barriers have not yet been investigated systematically; only the Mount Vernon fault has been well studied.

### Table 3. Results of Mantel tests (10000 permutations) of association between geographic distance and population pairwise FST within and among groups from hypotheses I and III, containing the same 15 northern MP-II populations partitioned in different ways.

<table>
<thead>
<tr>
<th>Hypothesis (group #)</th>
<th>Populations included</th>
<th>% variation explained by geographic distance</th>
<th>(P_{obs}&gt;sim(\alpha=0.05))</th>
</tr>
</thead>
<tbody>
<tr>
<td>III (1)</td>
<td>CLF, HIC, LAI, MOR, HIS, SRI, JGR, LAK, CLI</td>
<td>&lt;1</td>
<td>0.468</td>
</tr>
<tr>
<td>III (2)</td>
<td>FLE, PHC, SOR</td>
<td>&lt;1</td>
<td>0.6637</td>
</tr>
<tr>
<td>III (5)</td>
<td>TEA, MUL, GSP</td>
<td>&lt;1</td>
<td>0.673</td>
</tr>
<tr>
<td>I (1)</td>
<td>CLF, HIC, LAI, MOR, HIS</td>
<td>7</td>
<td>0.761</td>
</tr>
<tr>
<td>I (2)</td>
<td>SRI, JGR, LAK, CLI, MUL, GSP, TEA, SOR, PHC, FLE</td>
<td>19</td>
<td><strong>0.0035</strong></td>
</tr>
<tr>
<td>all northern MP-II</td>
<td>CLF, HIC, LAI, MOR, HIS, SRI, JGR, LAK, CLI, FLE, PHC, SOR, TEA, MUL, GSP</td>
<td>14</td>
<td><strong>0.0033</strong></td>
</tr>
<tr>
<td>all 27 populations</td>
<td></td>
<td>18</td>
<td><strong>0.0001</strong></td>
</tr>
</tbody>
</table>
The Mount Vernon fault (Fig. 4) runs through a cave-rich area of Rockcastle County, Kentucky. Based on its position in the otherwise relatively less faulted MP-II compared to other karst formations (KGS 2017), it may serve as a stratigraphic barrier isolating one of the three northern clusters (D. kentuckensis lexingtoni populations) from the other two (Fig. 5C red colored pie #1 and Fig 6C). The relatively cave-poor divide between the Kentucky and Rockcastle river drainages (KGS 2017), hypothesized by Barr (1985) to represent an important stratigraphic barrier, is not supported in this study given that populations of the northernmost genetic cluster fall on both sides of the barrier. The influence of the three-way fluvial barrier proposed by Barr (1985), formed by the confluence of the Cumberland River and its Big South Fork, is not explicitly supported but cannot be ruled out due to lack of breadth and spatial resolution in population sampling. Examination of geographically proximate populations in each sector of this “river triangle” (Barr 1985, see fig.1b in Kane et al. 1992) would help to clarify its role as an isolating barrier. Though our study did not explicitly test the effect of meander frequency (Barr 1985) on terrestrial troglobiont dispersal potential, the distributional patterns observed (Fig. 4) do not conflict with the hypothesis that smaller, meandering waterways are less likely or even unlikely to act as dispersal barriers compared to large rivers.

The sampling scheme of our study makes it difficult to extricate signal due to population structure from that due to IBD for the two genetic clusters on either side of the Cumberland River, which are strongly clustered spatially (Fig. 4). An ideal scheme would evenly sample many population pairs on either side of and at increasing distances from each proposed barrier. Under this sampling regime, results of partial Mantel tests within and among groups separated by each proposed barrier could be used to detect population structure amid underlying “noise” from IBD. Isolation between groups across fluvial barriers with different calculated meander frequencies could also be formally compared. Such a systematic sampling method would be challenging for this group of organisms however, as caves are unevenly distributed across the landscape and access restrictions further reduce the number of available cave sampling localities.

Overall, the limits of neither major nor minor watersheds alone adequately model the observed distribution of genetic diversity across sampled populations of D. kentuckensis. Geographic distance and landscape features, both stratigraphic and fluvial, appear to have each contributed to this distribution. Determination of the boundaries of cryptic species or subspecies, inference of their pattern of relatedness, and identification of predictive characteristics of isolating barriers will require further sampling of additional populations and more complete and/or additional molecular loci.

**Conclusion**

Based on CO1 data alone, there is a wide range of divergence values between taxa that can be defined as separate species on their own evolutionary trajectory from oth-
Geographically structured genetic diversity in the cave beetle Darlingtonia
er lineages (Hebert et al. 2003). No formal taxonomic changes are proposed herein as
a result of this study, as full or nascent species could be represented by all, some, or
none of the five genetic clusters discovered among twenty-seven sampled populations
of *D. kentuckensis*, depending upon the species definition favored. Both genetic and
some morphological evidence supports the hypothesis that *D. kentuckensis* consists of
isolated populations that could be considered as separate cryptic species or perhaps
subspecies. Hebert et al. (2003) gives an average sequence divergence of 11.2% be-
tween species of beetles within the same genus, but divergence ranges from below 1%
to 16–32% depending upon the paired taxa examined. Genetic divergence between
each of the five populations studied herein differ by ~1.3%, a percentage that is with-
in the range of CO1 sequence divergence between species, although it is certainly on
the low side. Regardless, populations within the range of the subspecies *D. kentuck-
ensis lexingtoni* do form a genetically distinct cluster that is especially supported by
this study; additionally all three northernmost clusters are geographically proximate
but genetically distinct, with little evidence that isolation by distance is an influence
on the pattern of genetic structure. The observed strong correlation between pairwise
*F*_*ST* and geographic distance among the two southern populations may either be an
artifact of sampling deficiency that overlooks intermediate haplotypes or a reflection
of a real historical sequence of colonization events. Therefore, these results can be
viewed as a starting point for continued investigation, using additional molecular
markers and denser sampling, of the historical phylogeography and species limits in
this group and other related taxa.

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References


Geographically structured genetic diversity in the cave beetle *Darlingtonea*


A subterranean species of *Exocelina* diving beetle from the Malay Peninsula filling a 4,000 km distribution gap between Melanesia and southern China

Michael Balke¹, Ignacio Ribera²

¹ SNSB-Zoologische Staatsammlung, München, Germany ² Institute of Evolutionary Biology (CSIC-Universitat Pompeu Fabra), Barcelona, Spain

Corresponding author: Michael Balke (balke.m@snsb.de)

Abstract

We describe a new subterranean species of the genus *Exocelina* Broun, 1886 (Coleoptera: Dytiscidae) from the Malay Peninsula. Almost all of the 196 species of that genus are epigean and distributed mainly in New Guinea, Australia, Oceania and New Caledonia. One epigean species is, however, known from China. The discovery of a species on the Malay Peninsula fills that distribution gap to some degree.

Keywords

Beetles, blind subterranean species, disjunct distribution, new species

Introduction

Here we report the discovery of a new subterranean diving beetle from the Malay Peninsula. This species was placed in the Dytiscidae, subfamily Copelatinae based on morphological characters using the key of Miller and Bergsten (2016). It was then unambiguously assigned to the genus *Exocelina* Broun, 1886 in a phylogenetic analysis using molecular systematic data of Toussaint et al. (2014, 2015, 2020 in preparation).

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The 196 described species of *Exocelina* are mostly from New Guinea (141 species, see e.g. Balke 1998; Shaverdo et al. 2018, 2019; Shaverdo and Balke 2019), followed by New Caledonia (37 species) and Australia (16 species, two of them subterranean), with single species each in Hawaii and Vanuatu (Balke et al. 2007; Nilsson and Hájek 2019). All of these localities lie east of the Lydekkers line. A single species was discovered in Shizong, Yunnan, China (Balke and Bergsten 2003), leaving a gap of around 4,000 km in the distributional range of *Exocelina*, essentially the entire Indonesian Archipelago and mainland Southeast Asia. The present finding partly fills this gap and suggests that more discoveries are to be expected, for example from the little sampled mountain regions of Vietnam and Laos. A synopsis of the subterranean diving beetles of the World was provided by Miller and Bergsten (2016), who provide an identification key as well as habitus photographs.

**Material and methods**

Specimens were studied with a Leica M205C stereo microscope at 10–160x. Images were taken with a Canon EOS 5DS camera fitted with a Mitutoyo 10x ELWD Plan Apo objective attached to a Carl Zeiss Jena Sonnar 3.5 / 135 MC as focus lens. Illumination was with two to four LED segments SN-1 from Stonemaster (https://www.stonemaster-onlineshop.de). Image stacks were generated using the Stackmaster macro rail (Stonemaster), and images were then assembled with the computer software Helicon Focus 4.77TM.

Drawings were produced with a camera lucida, first sketched with pencil on paper, then photographed and digitally inked using an iPad Pro and the Concepts as well as MediBang Paint APPs.

One paratype male of the new species (voucher number IBE-AN1160) was used for a non-destructive DNA extraction using a commercial kit (Qiagen DNeasy Tissue Kit). We successfully amplified six mitochondrial and nuclear genes in five sequencing reactions, two cytochrome c oxidase subunit I fragments (COI-5′ - the "barcode"- and COI-3′), 5′ end of rrnL RNA plus leucine tRNA transfer (tRNA-L1) plus 5′ end of NADH dehydrogenase subunit I (NAD1), and one internal fragment of both small ribosomal unit (18S RNA) and Histone 3 (H3) (see Villastrigo et al. 2018, for details of the primers and sequencing conditions). These are fragments routinely used for Dytiscidae systematics. Sequences were edited using Geneious v10.1 (Kearse et al. 2012). Here, we combined the newly obtained sequences of COI-3′, 18S and H3 (ENA database with accession numbers LR759936 H3, LR759937 18S, LR759938 3′COI, LR760127 5′COI) with the data of Toussaint et al. (2014, 2015 as well as 2020 in preparation). Other markers used by the latter authors (such as Carbomoylphosphate synthase (CAD) and Alpha-Spectrin (Asp)) could not be amplified here.

The combined dataset was analysed with a fast maximum likelihood search as implemented in IQ-TREE v1.6 (Nguyen et al. 2015), with a partition by gene fragment and the best evolutionary model as selected by Modelfinder (Kalyaanamoorthy et al. 2017) using the AIC (Akaike Information Criterion). We assessed topological stability
with 1000 ultrafast bootstraps and tested tree branches by SH-like aLRT with 1000 replicates (Nguyen et al. 2015).

Repositories

IBE Institute of Evolutionary Biology, Barcelona, Spain
KSc Kazuki Sugaya collection, Zama, Japan
NMW Naturhistorisches Museum Wien, Austria
ZSM Zoologische Staatssammlung München, München, Germany

Taxonomy

Family Dytiscidae Leach, 1815
Genus Exocelina Broun, 1886

Exocelina sugayai sp. nov.
http://zoobank.org/D7A59208-6691-4E3E-8899-9942AC745D4A

Type locality. Malaysia, Pahang, Cameron Highlands, Tanah Rata, 4.474705, 101.384043.


Paratypes: 4 males (1 used for DNA extraction and sequencing, voucher No. IBE-AN1160) and 2 females, same label data as holotype (IBE, KSc, NMW, ZSM).

Description of holotype. Size and shape: Smallest Exocelina known (length of holotype including head 2.7 mm, length without head 2.4 mm, greatest width 1.0 mm). Abdomen comparably parallel sided; pronotum also comparably parallel sided, slightly constricted before base, hind angles produced backwards (Fig. 1A).

Coloration. Testaceous and slightly translucent (Figs 1A, B, 2A–F).

Surface sculpture. Head and pronotum with distinct microreticulation formed by small regular cells and fine moderately dense punctuation. Elytra with distinct microreticulation formed by small regular cells and dense, coarse, setiferous punctuation (Fig. 1A, D). Ventral side with distinct microreticulation formed by small regular cells, including distinct microreticulation on metacoxal processes (Figs 3A, 4A–C).

Structures. Eyes fully reduced, with only small black scars remaining on surface of head (Figs 1A, B, 2A, B). Male antennomeres strongly modified: 2 and 3 moniliform, 4 slightly broadened in dorsal view, 5–11 strongly expanded, 11 flat and blade like (Fig. 1A). Fore tarsus dilated, fore angle of tarsomere 4 ventrally produced (Fig. 1C) and with two thicker setae (but no hook as in other Exocelina), on tarsomere 5 ventrally without obvious setation; pro and mesotarsomeres 1–3 with 4 rows of stalked suction discs (2 per row). Pronotum with faint lateral bead not reaching an-
Figure 1. *Exocelina sugayai* sp. nov. A habitus dorsal of male B same of female C foretarsus of male, arrow pointing at expanded anterior ventral angle of tarsomere IV D surface sculpture on male elytral disc, cropped from A. Length of left beetle: 2.7 mm.

Anterior nor posterior corners (Fig. 2B, D, F). Prosternal process short, lanceolate, deflexed, gently rounded ventrally (Figs 3A, 4A); metaventrite broadly triangular, its lateral “wings” very narrow (Fig. 4B, C). Membranous wings strongly reduced, with only very short stubs visible at the wing base. Metacoxal “lines” broadly diverging, fainting well before hind margin of metaventrite (Figs 3A, 4B). Metacoxal processes small, more elongate oval, with wide gap in middle (to possibly enable higher mobility of hindlegs) (Figs 3A, 4B). Last ventrite apically rounded. Median lobe of aedeagus simply curved in lateral view, parameres of simple, Copelatinae-type triangular shape (Fig. 5A, B).

**Female.** Antennomeres filiform to slightly moniliform (Fig. 1B). Pro and mesotarsomeres 1–3 not bearing stalked suction discs and protarsomere 4 not modified.
Figure 2. *Exocelina sugayai* sp. nov. male

A eye in lateral view
B detail of head and pronotum
C surface sculpture on base of head and anterior margin of pronotum
D detail of posterior angle of pronotum
E detail of surface sculpture on base of elytron
F detail of lateral view of elytral and pronotal base and head.
Variation. Length of beetle including head 2.4–2.8 mm. Two paratypes are darker orange (see Fig. 1B). According to the collector, this is due to subsequent darkening in alcohol storage.

Etymology. Named after Kazuki Sugaya, the discoverer of this species.

Differential diagnosis. This species differs from all other Dytiscidae by: Copelatinae with reduced eyes; beetle length < 3 mm; body with well visible microreticulation; prosternal process short and deflexed; metacoxal processes small, more elongate oval (in other Copelatinae, including the groundwater species *Exocelina abdita* Balke et al. 2004, this structure is more rounded, and the metacoxal “lines” can be more parallel sided, Figs 3B, 4D); male with strongly modified antennomeres.
**Figure 4.** *Exocelina sugayai* sp. nov. male, ventral side **A** prosternal process and mesocoxal area **B** metacoxa and metacoxal processes **C** metaventrite and metaxoxa **D** *Exocelina abdita*, metacoxa and metacoxal processes. Lines in **B** and **D** inserted to highlight outline of metacoxal processes.

**Habitat.** Collected from two helocrenes on a slope in forested area. The beetles were observed creeping around and were not swimming when observed (K. Sugaya personal communication 2019) (Fig. 6A, B).

**Phylogenetic affinities.** The best evolutionary model fitting the data according to Modelfinder was a GTR+F for all partitions. *Exocelina sugayai* sp. nov. was recovered deeply subordinated within *Exocelina*, as the sister of the Chinese *E. shizong* Balke &
Bergsten, 2003 and the New Caledonian *E. nehoue* Balke et al., 2014. These three species are part of a clade (“C4” in Toussaint et al. 2015) otherwise containing *E. parvula* (Boisduval, 1835) from Hawaii as well as a clade of New Caledonian and one Vanuatu species (Fig. 7). The other two subterranean species of *Exocelina* are *E. abdita* Balke et al., 2004 and *E. rasjadi* Watts & Humphreys, 2009 from Australia. The former was included in our phylogenetic analysis and placed in a different clade than *Exocelina sugayai* sp. nov. (Fig. 7, included subterranean species in red). Data for *E. rasjadi* were not available.

**Discussion**

Most species of *Exocelina* inhabit stream associated (lotic) habitats, specifically areas of stagnant water at the edge of streams and creeks, the interstitial and tiniest of water holes on riverbanks, as well as small puddles in intermittent creeks including the source area that might only have occasional water flow after rainfalls (see habitat photos in Shaverdo et al. 2012). This is the likely ancestral habitat type in *Exocelina*, with four subsequent shifts to lentic habitats (and only a few species in the lentic clades) (Toussaint et al. 2015). Most species have limited geographic ranges; in one widespread epigean species population genomic studies revealed strong geographic structure even in populations as close to each other as 40 km straight line (Lam et al. 2018).
New subterranean diving beetle

Figure 6. Habitat of *Exocelina sugayai* sp. nov. **A** overview **B** detailed, with a beetle crawling about in the center of the image.
Figure 7. Simplified phylogenetic tree obtained with IQ-TREE using the DNA sequence dataset of Toussaint et al. (2014, 2015 as well as 2020 in preparation) plus the newly obtained sequences of *Exocelina sugayai* sp. nov. Non-relevant clades are collapsed to genus or other major clades. Numbers in nodes, ultrafast bootstrap / SH-like aLRT support.

The lotic beetles often hide in the gravel when disturbed, and observations of M. Balke in New Guinea suggest that the interstitial of riverbanks is often utilized by these beetles, possibly to avoid downstream drift. The beetles seem to avoid habitat with fine, dense substrates, which we suggest make it hard to hide as such substrate clogs the space between stones and pebbles (see also Balke 2001).
This lifestyle could be interpreted as a preadaptation for interstitial or stygobitic life. In fact, some Australian species seem to mainly inhabit the interstitial, and have been suggested to provide a scenario for the transition from epigean to stygobitic life (Watts et al. 2016). To date, two species have been described from groundwater habitats in Australia. They exhibit a strongly modified morphology typical of stygobitic species, such as wing and eye reduction and depigmentation (Balke et al. 2004; Watts and Humphreys 2009, see also Watts et al. 2016). The discovery of the new species described here suggests that many more such stygobitic *Exocelina* could be found in the future. Our phylogenetic analysis also suggests that the evolution of subterranean *Exocelina* occurred at least two times independently (Fig. 7). In Copelatinae, one species of the genus *Copelatus* Erichson, 1832 from Brazil has been described from the subterranean habitat (Caetano et al. 2013).

Biogeographically, the occurrence of Southeast Asian and a Chinese species of *Exocelina* remains enigmatic. The origin of the clade containing these species was estimated as at least 10 million years ago (“C4” Toussaint et al. 2015). Based on the information currently available, we can not state with confidence whether the Asian species are “relics” of a previously diverse and widespread *Exocelina* fauna, or the result of rare dispersal events without apparent subsequent diversification.

**Acknowledgements**

We express our sincere thanks to Kazuki Sugaya for sending the specimens studied here to the senior author, and Anabela Cardoso for laboratory work. Helena Shaverdo and Günther Wewalka (Vienna) provided very valuable reviews of the submitted manuscript. This research was supported by DFG Ba2152/4-1, 7-1, 11-1, 11-2 and 24-1. Michael Balke acknowledges support from the EU SYNTHESYS program projects FR-TAF-6972 and GB-TAF-6776.

**References**


New subterranean diving beetle


Two new dipluran species unearthed from subterranean habitats of the Canary Islands (Arthropoda, Hexapoda, Entognatha)

Alberto Sendra¹, Heriberto López², Jesús Selfa³, Pedro Oromí⁴


Corresponding author: Alberto Sendra (alberto.sendra@uv.es)

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http://zoobank.org/B2058907-20E7-465A-8F34-1FA674D9BB6F


Abstract

Two new dipluran species of the family Campodeidae have been unearthed in the Canary Islands. Remycampa herbanica sp. nov. was found in a highly threatened lava tube on Fuerteventura island. It is related to the soil-dwelling northwest African Remycampa launeyi that also inhabits four of the Canary Islands. The two known Remycampa species are characterized by a torsion of the labial palps. They differ chiefly in the distribution of macrosetae and in the features of cave adaptation of R. herbanica, i.e. elongation of body and appendages, and a higher number of olfactory chemoreceptors with a coniform shape unique within campodeids. Spaniocampa relicta sp. nov. was collected in the mesovoid shallow substratum (MSS) and has been assigned to a formerly monotypic genus that includes the soil-dwelling Spaniocampa prima from the Republic of Guinea. The two species differ in the number of abdominal macrosetae. Females of S. relicta sp. nov. have small setae arranged in groups along the posterior border of the first urosternite. These structures of unknown function have never been described in other campodeid species. Sequencing the COI barcode region of R. herbanica has been produced but it proved insufficient to identify closest relatives. The two new hexapods from subterranean habitats raise the Canarian campodeid fauna to six species. Five of them are living in soil and/or MSS, whereas the cave-adapted R. herbanica is known only from a single, particularly endangered lava tube distant from other caves.
Keywords
Campodeidae, cave-adapted fauna, DNA barcoding, mesovoid shallow substratum, new species, Remycampa, Spaniocampa

Introduction

With almost 1000 known species, Diplura are the second most diverse Entognatha after Collembola (Deharveng and Bedos 2018). All Entognatha like diplurans are good examples of successful colonizers of hypogean habitats, thriving in all kinds of cryptic environments without light (Condé 1955; Racovitza 1907), including caves reaching the deepest habitats in the continental crust (Sendra et al. 2020). Furthermore, diplurans have a series of regressive adaptive features common to cave-dwelling animals, given their thin and almost completely unpigmented cuticle and absence of external eyes. However, they have remnants of lateral sense organs, each lying below the integument at both sides of the head in latero-ventral position, which presumably have a light-perceptive function (George 1963). Diplurans are divided into ten families, Campodeidae and Japygidae having the lion’s share of all species in the group (Paclt 1957; Pagés 1959; 1989; Rusek 1982; Sendra 2015). The two aforementioned families and the smaller Parajapygidae have already been recorded on most of the Canary Islands (Paclt and Báez 1990, 1992; Pagés 1993; Sendra 1989, 1990; Sendra and Báez 1986). So far, a total of four Campodeidae, two Japygidae and one Parajapygidae species have been found mainly in soil habitats of this archipelago. We focused the present study in the Canaries on the lesser known subsurface habitats, i.e. the volcanic caves and the “Milieu Souterrain Superficiel” (hereafter MSS) (Juberthie et al. 1980) rather than the soil itself.

Most of the volcanic cavities are lava tubes, which usually lie a few meters below ground due to their particular origin from surface flowing lavas (Wood and Mills 1977; Wood 1979), therefore considered as part of the Shallow Subterranean Habitats (hereafter SSH) (Culver and Pipan 2014), defined as a set of mixed habitats just below the surface (soil, MSS and lava tubes among the terrestrial habitats). In spite of being relatively shallow, in volcanic terrains both lava tubes and the MSS often hold interesting cave-dwelling fauna comparable to that adapted to deeper continental karstic caves (Howarth 2008; Oromí and Martín 1992). However, no important cave-adapted species of Diplura have been found in either lava tubes or the MSS of the Canaries or Hawaii, the richest volcanic archipelagos for cave animals. Some lava tubes can occasionally be located deeper, covered by several layers of younger lava flows and commonly devoid of fauna due to the difficulty to organic matter reaching such depths. Only a few known cases of really deep tubes are suitable for adapted fauna, like the 14 million years old Cueva de Aslobas, in the south-west of Gran Canaria island (Fernández et al. 2015). Cave-adapted animals are also absent from most lava tubes in very dry areas, such as in most of the semi-arid eastern Canary Islands, with only two exceptions on Fuerteventura: Cueva del Llano and Cueva de Montaña Blanca (Rando et al. 1993; Naranjo and
Oromí 2011) (Figs 1–3). The Canary Islands lava tubes have no permanent water flow inside, making soil accumulation scarce, which may limit the abundance of diplurans.

Another important SSH just below the edaphic layers (i.e. soil) is the “milieu souterrain superficiel” formerly described by Juberthie et al. (1980) for non-calcareous areas of
the French Pyrenees, and later named by Culver and Pipan (2010) as “mesovoid shallow substratum” (MSS). There are different kinds of MSS, depending on the rock composition and geomorphologic origin, defined as a habitat representing the underground network of empty air-filled voids and cracks developing within multiple layers of rock fragments (Mammola et al. 2016; Ortuño et al. 2013). The MSS is usually covered by topsoil, connected with underlying deep rock cracks and caves. Fauna in MSS has been successfully surveyed in the Canaries, mostly in the typical colluvial MSS from talus deposits similar to those in continental non-volcanic terrains (Medina and Oromí 1990; Mammola et al. 2016), and in the peculiar volcanic MSS formed by lava clinker covered by a layer of protective soil (Oromí et al. 1986; Pipan et al. 2010). The latter is very abundant in recent and subrecent terrains (a few hundred thousand years) on most islands of the archipelago, providing a widespread subsurface habitat present in areas with or without lava tubes. The MSS in these islands has turned out to be almost as rich in cave-adapted fauna as the caves themselves. The few unidentified diplurans previously collected in such environments were always in colluvial MSS in the older parts of Tenerife and La Gomera, which is richer in soil and organic matter than the younger volcanic MSS (Medina and Oromí 1990, Pipan et al. 2010). Further sampling in the MSS of Gran Canaria (Fig. 4) and in an old cave on Fuerteventura has provided the new material of Campodeidae diplurans studied herein.

Material and methods

Sampling and imaging

Specimens from Fuerteventura were collected in Cueva de Montaña Blanca (Figs 1–3) using pitfall traps with propylene glycol as preservative and blue cheese as bait, and sometimes just cheese on the ground to attract them, for live collection. Specimens from Gran Canaria were collected in the MSS at Brezal del Palmital (Fig. 4) using the pitfall traps described by López and Oromí (2010), baited with raw liver or cheese and with propylene glycol as preservative. The individuals were stored in ethanol (70–75%), washed with distilled water, mounted on a slide with Marc André II solution, and examined under a phase-contrast optical microscope (Leica DMLS). The illustrations were made with a drawing tube, and measurements taken with an ocular micrometer. To determine body length, specimens were mounted in toto and measured from the base of the distal macrochaetae on the frontal process to the abdominal supra-anal valve. Two specimens from Cueva de Montaña Blanca coated with palladium-gold were used for SEM photography (Hitachi S-4800) and for measurements of the sensilla.

Morphological study

The morphological descriptions and abbreviations are following Condé (1955). We use the term gouge sensilla for the concavo-convexly shaped sensilla on the anten-
DNA extraction, PCR ampand sequencing

Sequences of the 5’ end of the cytochrome c oxidase subunit I (COI), a DNA fragment considered the standard DNA barcode region for Metazoa (Hebert et al. 2003),
were generated for one of the specimens collected on Fuerteventura. For this, genomic DNA was extracted using the DNeasy Tissue Kit (Qiagen) following the manufacturer's guidelines. Amplification by PCR was done using the primers LCO1490 and HCO2198 (Folmer et al. 1994) in a 25 μl total PCR volume containing 15.4 μl of purified water, 2.5 μl of 10x NH₄-based Reaction Buffer, 1.5 μl of MgCl₂ (3mM), 2 μl of 10 mM dNTP (2.5 mM each), 0.5 μl of BSA, 1 μl of each primer (10 μM), 0.1 μl of BIOTAQ™ DNA polymerase, and 1 μl of DNA extract. The PCR was executed with the following protocol: initial denaturing step at 95 °C for 2 min, 40 amplification cycles (94 °C for 30 s, 46 °C for 35 s, 72 °C for 45 s), and a final step at 72 °C for 5 min. PCR success was checked by running products on a 1% TAE agarose gel. Successfully amplified products were cleaned following EXO I/rAP PCR clean-up protocol and outsourced for DNA sequencing by Macrogen Inc. (https://dna.macrogen.com).

**Depositories**

The material examined is deposited in the following collections:

- **ASM**  
  Personal collection of Alberto Sendra, Valencia, Spain
- **IPNA-CSIC**  
  Invertebrates collection of the Instituto de Productos Naturales y Agrobiología (IPNA-CSIC), Tenerife, Canary Islands, Spain
- **MCNT**  
  Museum of Natural History of Tenerife, Canary Islands, Spain
- **DZUL**  
  Collection of the Department of Animal Biology, University of La Laguna, Canary Islands, Spain

**Results**

**Taxonomic acts**

*Subphylum Hexapoda* Blainville, 1816  
*Class Entognatha* Grassi, 1889  
*Order Diplura* Börner, 1904  
*Suborder Rhabdura* Cook, 1896  
*Family Campodeidae* Lubbock, 1873  
*Subfamily Campodeinae* Condé, 1956

*Remycampa herbanica* Sendra & Oromí, sp. nov.  
http://zoobank.org/5619DB84-4E4A-4293-85E7-3C6A65B9F392  
Figs 5–30; Tables 1, 2

**Type locality.** Spain, Canary Islands, Fuerteventura: El Castillo, Montaña Blanca Cave (28°24’3.48”N, 13°52’51.08”W, 166 m a.s.l.).
Figures 5–12. *Remycampa herbanica* sp. nov. 5 Distal antennomere 6 lateral detail of the cupuliform organ with olfactory chemoreceptors 7 cupuliform organ 8 apical end of an olfactory chemoreceptor 9 medial antennomere 10 gouge sensilla 11 frontal process 12 ventral view of the head, detail of labial palps and submentum.
**Type material.** Holotype: 1 ♀, Spain, Canary Islands, Fuerteventura: El Castillo, Montaña Blanca Cave (28°24'3.48"N, 13°52'51.08"W, 166 m a.s.l.), 5 October 2018, A. Sendra & P. Oromí leg. (DZUL). Paratypes: 5 ♂♂, 1 juvenile (labelled M1 to M5-paratype and J-paratype), same locality as holotype, 12 July 2015, P. Oromí, H. López & B. Rodríguez leg. All type material mounted in Marc André II solution. Depositories: DZUL (2 ♂♂), IPNA-CSIC (1 ♂), ASM (2 ♂♂, 1 juvenile).

**Other studied material.** Same data as holotype, two specimens mounted on two separate aluminium stages and coated with palladium-gold.

**Description.** Body length 3.8–4.4 mm in males (n = 5), 4.2 mm in females (n = 1) and 2.2 mm in one juvenile (Table 1). Epicuticle smooth under optical microscope but slightly reticulated at high magnifications as irregular polygonal structures of variable size (Fig. 14). Body with scarce short clothing setae with one or two apical barbs on each seta (Fig. 18).

Antennae with 36 antennomeres in one complete intact antenna in the holotype; antennae 0.84× as long as the body length with medial antennomeres 2× longer than wide, as is the apical antennomere. Cupuliform organ with about 21 complex olfactory chemoreceptors arranged in two concentric circles with one in the centre, each apparently with a pile of fused plates forming a coniform structure (Figs 5–9). Distal and central antennomeres with two or three whorls of barbed macrosetae and scattered smooth setae, in addition to a single distal whorl of 8–12 short thick gouge sensilla 10 μm long (Fig. 10). These latter are more abundant on the dorsal side of the antennomere, including one or two very short coniform sensilla. Proximal antennomeres with typical trichobothria, plus a small coniform sensillum on third antennomere in ventral position.

Moderate protrusion of frontal process covered with very slightly tuberculated setae with two to five barbs on distal half (Fig. 11). Three macrosetae along each side of the line of insertion of antennomere and setae x with thin distal barbs; length ratios al/i/p/x as the 29/26/17/24 in female paratype (Fig. 11).

Large mandibulae with at least five teeth, the two posterior ones with a row of small denticles. Atypical labium with slight torsion to the right of the labial palps, slight elongation of the palpiform processes, and a deep groove in the middle of labium from posterior border of anterior lobe to the middle of submentum, without reaching the posterior border of labium (Fig. 12). Suboval labial palps each with small latero-external sensillum, three guard setae and up to 68 neuroglandular setae (Fig. 12).

**Table 1.** *Remycampa herbanica* Sendra & Oromí, sp. nov. (all units in mm except number of antennomeres).

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Body length</th>
<th>Antennae length</th>
<th>Number of antennomeres</th>
<th>Metathoracic leg</th>
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<tbody>
<tr>
<td>Paratype, ♂ 1</td>
<td>4.4</td>
<td>–</td>
<td>–</td>
<td>Coxa 0.16, Trochanter 0.12, Femur 0.60, Tibia 0.76, Tarsus 0.53, Total leg 2.17</td>
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<td>3.54</td>
<td>36</td>
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<tr>
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<td>–</td>
<td>–</td>
<td>Coxa 0.18, Trochanter 0.12, Femur 0.52, Tibia 0.80, Tarsus 0.50, Total leg 2.12</td>
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<td>–</td>
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<tr>
<td>Paratype, J</td>
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<td>–</td>
<td>–</td>
<td>Coxa 0.10, Trochanter 0.08, Femur 0.36, Tibia 0.38, Tarsus 0.30, Total leg 0.92</td>
</tr>
</tbody>
</table>
Figures 13–16. *Remycampa herbanica* sp. nov. 13 Pro-, meso- and metanotum of holotype, left side 14 detail of pronotum with medial anterior macrosetae 15 left posterior portion of pronotum and left anterior anterior portion of mesonotum 16 right posterior portion of mesonotum with lateral posterior macrosetae.
Thoracic macroseta distribution (Figs 13–20): pronotum and mesonotum with 1+1 ma, 1+1 la, 1+1 lp macrosetae; metanotum with 1+1 ma macrosetae. All macrosetae short and slightly thick with short barbs along basal two-thirds of each seta; marginal setae longer and more barbed than clothing setae (Figs 13–20). Legs elongated, metathoracic legs reaching abdominal segment IX, about 0.5x as long as the body length (Figs 21–26; Table 1). Tibia always longer than femur or tarsus (Table 1). Femorae I–III each with one short thick dorsal macroseta with a few barbs. Calcars with long barbs throughout one side (Fig. 25). Tibiae I–III with two short ventral macrosetae with two to four distal barbs; some paratypes with three sternal tibial macrosetae on the metathoracic leg (Figs 23, 26). Two rows of ventral barbed setae with two lines each of two to five barbs (Figs 21, 25). Three smooth dorsal distal tarsal setae longer than the rest (Fig. 21). Subequal claws with a lateral expansion curved towards the two ventral sides. Smooth laminar telotarsal processes curved along and ending in a slightly wide expansion with a narrow prolongation on one side, a unique shape among diplurans (Figs 21, 22, 25).

Distribution of abdominal macrosetae on tergites (Fig. 27): 1+1 ma on I–III; 1+1 ma, 1+1 la on IV, 1+1 ma, 1+1 la, 1+1 lp on V–VII; 1+1 mp, 3+3 lp on VIII; and 1+1 mp, 5+5 lp on IX abdominal segment. All tergal abdominal macrosetae short, slightly thick with thin short barbs being ma and mp the shortest.
Two new diplurans in subterranean habitats of the Canary Islands

Figures 21–26. *Remycampa herbanica* sp. nov. metathoracic leg. 21 Distal portion of the tarsus 22 detail of claws 23 right metathoracic leg 24 pretarsus 25 joint between tibia and tarsus with a calcar 26 medial portion of tibia with ventral macrosetae.

Urosternite I with 6+6 macrosetae (Figs 28, 29); urosternites II to VII with 4+4 macrosetae; urosternite VIII with 1+1 macrosetae; urosternal macrosetae of medium length or longer, with a few long barbs in one single row along the distal half to four-fifths. Stylus with an apical, a subapical and a ventromedial seta with a few long barbs arranged in one row along the distal four-fifths (Fig. 30). Cerci more than 2× as long as the body length, 2.1× as long as the body in the only apparently intact cercus of the holotype; with 27 primary articles, not counting the multi-divided basal article (Table 2). Length
of cerci increases very slightly from the proximal to distal articles; they are covered with a whorl of alternate smooth thin macrosetae and smooth thin setae, and a whorl of shorter smooth thin setae at the end of each primary article. These whorls, except the apical one, increase from one to four from the proximal to distal primary articles.

Figures 27–30. *Remycampa herbanica* sp. nov. 27 Dorsal view of abdomen, right side, holotype 28 male first urosternite, paratype 29 female first urosternite 30 left stylus and vesicle of the fifth urosternite. *s* = setiform sensillum).
Two new diplurans in subterranean habitats of the Canary Islands

Table 2. *Remycampa herbanica* Sendra & Oromí, sp. nov. (all units in mm except number cercal articles and basal secondary articles).

<table>
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<th>Divisions basal article</th>
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<th>1st</th>
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</table>

Female urosternite I with slim cylindrical appendages, each bearing up to seven glandular a₁ setae in a distal field (Fig. 29).

Male urosternite I with short coniform appendages, each bearing about 13 glandular a₁ setae in a distal field; posterior edge occupied by a large but narrow field of cramped up to 190 glandular g₁ setae (Fig. 28).

**Etymology.** Referring to Herbania, the ancient name of Fuerteventura, the only island on which it has been found.

**Molecular data.** The barcode sequence of one specimen of *R. herbanica* (code 112BC) has been registered in GenBank with the ascension number MN729498.

**Phylogenetic analyses.** Available COI barcode sequences of Diplura stored in BOLD were retrieved (search for Diplura on 14th November 2019 at http://www.boldsystems.org/index.php/) to identify the species closest to *R. herbanica*. After excluding redundant sequences for several taxa, a total of 46 sequences, representing approximately 28 species from at least 10 genera were retained. They were then aligned with the newly generated *R. herbanica* sequence using the MAFFT E-INS-I algorithm (Katoh et al. 2002). A preliminary maximum likelihood tree was generated using the Fast Tree 2.1.5 (Price et al. 2009) tool in Geneious 7.1.9 (Kearse et al. 2012) to identify taxa closely related to *R. herbanica*.

The genetic results do not show well supported relationships of *R. herbanica* with the other diplurans with barcode sequences in BOLD. Based on this preliminary result we only can confirm genetically that this new species belongs to the family Campodeidae.

*Spaniocampa relicta* Sendra & López, sp. nov.
http://zoobank.org/588E7856-C77B-45F1-9D86-C476B4C37C1C
Figs 31–34

**Type locality.** Spain, Canary Islands, Gran Canaria: Brezal del Palmital (28°6’33.58”N, 15°36’1.73”W, 551 m a.s.l.).

**Type material.** Holotype: 1 ♀, Spain, Canary Islands, Gran Canaria: Brezal del Palmital (MSS3) (28°6’33.58”N, 15°36’1.73”W, 551 m a.s.l.), 4 July 2010, H. López leg (DZUL). Paratypes: same data as holotype, 1 ♀, 1♂ (ASM). All type material mounted in Marc André II solution.
Description. Body length 3.4 mm (paratype) and 4.1 mm (holotype) in females, and 3.5 mm (paratype) in male. Epicuticle with small microdenticles under optical microscope on dorsal side of nota and legs. Body with smooth clothing setae.

Broken antennae on the three types; medial antennomeres (antennomere XII intact) as long as wide, a single distal whorl of 8–10 short and thin gouge sensilla 12 μm long. Proximal antennomeres with typical trichobothria plus a bacilliform sensillum on third antennomere in ventral position.

Plain frontal process with one anterior and three posterior smooth setae; length ratios of a/p as 53/23 in holotype. The three macrosetae along each side of the line of insertion of antennomere with thin distal barbs and length ratios of a/i/p as 17/27/16 in holotype; no x setae observed; Each suboval labial palp has a small latero-external subcylindrical sensillum; two guard setae, up to three simple setae on anterior border and up to 70 neuroglandular setae in holotype.

Non-thoracic macrosetae on pronotum, mesonotum and metanotum; short slightly thick marginal setae with very thin or smooth apical barbs (Fig. 31). Metathoracic legs reaching abdominal segment VII. Femora without dorsal macrosetae. Tibiae I–III without typical ventral barbs but with a short ventral apical one with a few thin distal barbs; calcars with three to six thin barbs along one side. Two dorsal tarsal smooth setae similar to clothing setae, but much longer. Subequal claws, slightly wider at the base and regularly curved. Smooth setiform telotarsal processes overpassing the end of the claws.

Distribution of abdominal macrosetae on tergites (Fig. 34): 1+1 lp 3 on urotergite VIII; 3+3 lp 3,4,5 on abdominal segment IX, and 4+4 macrosetae on abdominal segment X; all these macrosetae long and well-differentiated with thin barbs along the distal third to three-quarters.

Urosternite I with 5+5 macrosetae; urosternites II to VII with 3+3 macrosetae; urosternite VIII with 1+1 macrosetae; urosternal macrosetae short to middle size with one to five apical to distal barbs (Figs 32, 33).

Stylus with an apical, a subapical and a ventromedial setae with a few distal thin long barbs, more abundant on the ventromedial seta (Fig. 33). Cerci absent in the studied specimens.

Female urosternite I with short subcylindrical appendages, each bearing up to 14 glandular a1 setae in a distal field. The posterior border of the urosternite bears three or four groups of small setiform setae with between two and ten units (Fig. 32).

Male urosternite I with short thick appendages, each bearing about 35 glandular a1 setae in two apparently distal fields; posterior edge slightly enlarged at both sides of the first urosternite with a glandular field of about 140 glandular g1 setae arranged in up to six rows.

Etymology. The specific epithet relicta refers to two situations affecting this new species: i) it has been discovered in a relict patch of laurel forest on Gran Canaria; ii) it is a relict species of a genus also distributed on the Republic of Guinea with one known extant species.
Figures 31–34. *Spaniocampa relicta* sp. nov. 31 Pro-, meso- and metanotum of holotype 32 female first urosternite, right side, paratype 33 fourth urosternite, right side, female paratype 34 eighth to tenth abdominal segments, ventral view, right side, holotype.
Discussion

Phyletic affinities

The substantially cave-adapted *Remycampa herbanica* sp. nov. is certainly related to the monotypic genus *Remycampa* Condé, 1952, due to several important taxonomic features including similarities in their atypical labium, secondary sexual characters, lateral telotarsal processes and distribution of macrosetae. The only species known so far, *Remycampa launeyi* Condé, 1953, has a distribution area in northeast Morocco and some of the Canary Islands (Sendra 1989), has extended to the islands of El Hierro, Tenerife, Gran Canaria, and Lanzarote. *R. herbanica* has been collected in a volcanic lava tube of Fuerteventura. Being only 11 km from Lanzarote one can postulate that *R. launeyi* might be present also in soils or MSS of Fuerteventura. Both islands were joined during the last glaciation (Fernández-Palacios et al. 2015) and had and have a similar climate. The most visible differences between *R. launeyi* and *R. herbanica* are in the cave-adapted features of the new species, which has a larger, more elongated body and appendages with cerci 2.1× longer than the body length and with 28 articles (Table 2), and with metathoracic tibiae bearing 2–3 sternal macrosetae. Furthermore, each apical antennomere has a large cupuliform organ with quite remarkably for the high number of olfactory chemoreceptors (up to 21) with a unique coniform shape (Figs 5–8). Other noteworthy morphological differences are: shorter and thicker macrosetae and shorter clothing setae with apical barbs in *R. herbanica*; differences in the shape of their lateral telotarsal processes, with trapezoidal endings in *R. launeyi* and round with a thin expansion in *R. herbanica* (Figs 21, 22, 24); Condé 1953: figures 3C, D and E); absence of lateral posterior macrosetae on metanotum in *R. herbanica*; absence of 1+1 lateral posterior macrosetae on third and fourth urotergites in *R. herbanica*; and finally differences between their labial pieces with a strong torsion to the right of labial palps and enlargement of the groove in the middle of the labium in *R. launeyi* compared with a less pronounced and smaller groove in *R. herbanica* (Fig. 12).

*Remycampa* is a peculiar genus with an unclear relation to other genera of Campodeinae, but with certain affinities with the tachycampoid phyletic lineage. It is probably more closely related to the two known cave-adapted tachycampoid genera living in caves of northwest Africa: *Jeannelicampa* Condé, 1952 from Oran in the Tell Atlas, Algeria, and *Tachycampa* Silvestri, 1936 from karst areas near Taza in the Middle Atlas, Morocco. Like *R. herbanica*, these two genera lack some thoracic macrosetae, short thoracic macrosetae and lateral expansions on the claws. Nevertheless, new taxonomic tools are needed to unravel the natural phylogenetic relations within Campodeinae and tachycampoid genera (Sendra et al. 2020).

It is difficult to determine the exact systematic position of *Spaniocampa relicta* sp. nov., not because of the broken antennae or missing cerci that cannot be described, but rather the lack of fresh specimens of the two closely related genera and their species. We refer to the monospecific *Spaniocampa* Silvestri, 1933 from Kakoulima massif (Republic of Guinea) and *Ombrocampa* Paclt, 1957 that, according to Paclt (1957), includes the three related species *O. dahli* Condé, 1956 and *O. nyongensis* Condé, 1956
from Nyong (Cameroon) and *O. depauperata* (Silvestri, 1918) from Mount Kenya (Kenya). They are all soil-dwelling, whereas *Spaniocampa relicta* was found in colluvial MSS. All these species have in common with *S. relicta* a low number of thoracic and abdominal macrosetae, including no dorsal macrosetae on femorae and no ventral ones on tibiae (with the exception of one short ventral tibial macroseta in *Spaniocampa prima* Silvestri, 1933). Furthermore, *S. prima* shares with *S. relicta* sp. nov. the total absence of notal macrosetae (Fig. 31) and similarities in the distribution of abdominal macrosetae: 2+2 lateral posterior macrosetae on eighth urotergite and ninth abdominal segment in *S. prima* and 1+1 lateral posterior on eighth urotergite and 3+3 lateral posterior on ninth abdominal segment in *S. relicta* (Fig. 34). Further differences to *S. prima* were also found in the number of urosternal setae, with: 8+8 macrosetae on first urosternite (this number could be reduced to 7+7, since Silvestri considered some barbed setae in latero-posterior position as macrosetae) and 4+4 macrosetae on second to seventh urosternites in *S. prima* compared with only 5+5 and 3+3 macrosetae in *S. relicta* sp. nov.

It is worth mentioning the presence of small setae arranged in groups on the posterior border of the first urosternite in females; their function is unknown, though apparently non-glandular, and they have never been described in any other species of the campodeid family.

**Dipluran fauna and their habitats**

The Canary Islands have a wide range of SSH in their volcanic landscapes: soils, MSS, and young and old lava tubes with a rich biodiversity (Oromí 2004). Diplurans had been collected in soil and MSS but not in lava tubes until now (Paclt and Báez 1990, 1992; Pagés 1993; Sendra 1989, 1990; Sendra and Báez 1986). Focusing on Campodeidae, six species of the subfamily Campodeinae are present in the Canary Islands. Two species of the genus *Campodea* are widespread in the Euromediterranean region and beyond: *Campodea* (*Campodea*) *fragilis* Meinert, 1865 and *Campodea* (*Mono-campa*) *devoniensis* Bagnall, 1918. Another two have more limited distribution areas: *Podocampa ceballosi* (Silvestri, 1932) in the Iberian Peninsula and northwest Africa, *Remycampa launeyi* Condé, 1952 limited to north-west Africa. The two new species *Spaniocampa relicta* sp. nov. and *Remycampa herbanica* sp. nov. are endemic to the Canaries. In relation with their habitats, *Campodea fragilis*, *C. devoniensis*, *Podocampa ceballosi* and *Remycampa launeyi* are frequently found in soil and are also present in the MSS as *Spaniocampa relicta* sp. nov. And, *Remycampa herbanica* sp. nov. is the only species occurring in lava tubes and showing cave-adapted features, also known as troglomorphic traits, as a result of its obligate lifestyle. It has been collected exclusively in Cueva de Montaña Blanca, one of the few lava tubes on Fuerteventura suitable for such adapted fauna (Figs 1–3). The presence of cave-adapted diplurans in other lava tubes around the world is not uncommon. Ferguson (1992) provided many localities from the USA, and Borges and Oromí (1994) reported the presence of one species in Gruta do Esqueleto, São Miguel island, Azores. Sendra et al. (2016) described a spe-
cies from Mexican volcanic caves, and an interesting cave-adapted *Lepidocampa* was reported from Reunion in the Indian Ocean (Sendra et al. 2017).

The special case of Cueva Blanca

Fuerteventura has a maximum sub-aerial age of 22 Ma, an exceptional span for a volcanic island, probably due to its extremely slow subsidence into the sea, compared to other volcanic archipelagos (Fernández-Palacios et al. 2011). For this reason, together with its scarce volcanic activity over the last million years, the island is highly eroded and most of the extant caves are dry and often silted with clay, thus being unsuitable to hold adapted troglobiont fauna. Only two of these lava tubes (Cueva del Llano and Cueva de Montaña Blanca, 27 km apart from each other) have appropriate humidity conditions for this fauna that includes eight troglobiont arthropod species which are all endemic to the island and often with no related species in the archipelago. Only the nicoletiid Zygentoma *Coletinia majorensis* Molero, Gaju, López, Oromí & Bach, 2013 inhabits both caves, the remaining seven species being exclusive to one or the other (Rambla 1993; Molero et al. 2013). The habitat of both caves is highly threatened. Cueva del Llano is a show cave owned by the local government, and many houses are built on the surface surrounding the cave, in spite of the exclusive presence of the officially protected harvestman *Maiorerus randoi* Rambla, 1993. Cueva de Montaña Blanca is the only known place where *Remycampa herbanica* sp. nov. and some undescribed troglobiont invertebrates occur (two weevils, one pseudoscorpion and one spider), and its entrance is within an unfinished abandoned four-story building in a tourist resort. The situation is critical for these cave-dwelling species given that most of the Fuerteventura underground is very dry, there is hardly any area of MSS, and therefore their inhabitable environment is highly limited to small distantly dispersed spots.

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Two new diplurans in subterranean habitats of the Canary Islands


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Two new diplurans in subterranean habitats of the Canary Islands


Cave-dwelling pseudoscorpions of China with descriptions of four new hypogean species of *Parobisium* (Pseudoscorpiones, Neobisiidae) from Guizhou Province

Zegang Feng¹, J. Judson Wynne², Feng Zhang¹

¹ The Key Laboratory of Zoological Systematics and Application, College of Life Sciences, Hebei University, Baoding, Hebei 071002, China ² Department of Biological Sciences, Colorado Plateau Museum of Arthropod Biodiversity and Merriam-Powell Center for Environmental Research, Northern Arizona University, Flagstaff, Arizona 86011, USA

Corresponding author: Feng Zhang (dudu06042001@163.com)

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Abstract

We summarize and discuss the 29 known cave-dwelling pseudoscorpion species from China. Four new troglobiont pseudoscorpion species, *Parobisium motianense* sp. nov., *P. qiangzhuang* sp. nov., *P. sanlouense* sp. nov., and *P. tiani* sp. nov., belonging to the family Neobisiidae, are described based on specimens collected in karst caves in Guizhou, China. Detailed diagnosis, descriptions, and illustrations are presented. We also provide recommendations for management of caves where they occur, as well as the cave arthropod communities and the habitats that support them.

Keywords
cavernicoles, cave conservation, taxonomy, troglobionts
Introduction

Biospeleological studies in the South China Karst (SCK) has rapidly accelerated in recent years. Since 2017, 39 new subterranean-adapted species across several taxonomic arthropod groups have been described (Gao et al. 2017; Huang et al. 2017; Li and Wang 2017; Song et al. 2017; Tian et al. 2017, 2018; Deuve and Tian 2018; Li et al. 2019a). Overall, at least 382 cave-dwelling arthropod species are now known from this region (Ran and Yang 2015; Tian et al. 2016; Li and Wang 2017; Gao et al. 2018; Feng et al. 2019; Li et al. 2019b; Liu and Wynne 2019). Incidentally, this work has also resulted in the identification of at least 21 troglomorphic pseudoscorpion species (refer to Feng et al. 2019; Li et al. 2019b).

In the last 25 years, cave-dwelling pseudoscorpions from China, specifically in Guizhou, Yunnan, Guangxi, Sichuan, and Hubei Provinces, and Beijing and Chongqing Municipalities, total at least 29 pseudoscorpion species (Schawaller 1995; Mahnert 2003, 2009; Mahnert and Li 2016; Gao et al. 2017; Li et al. 2017; Gao et al. 2018; Feng et al. 2019; Li et al. 2019; Table 1). Of these, 23 are troglobionts and six are troglophiles (Table 1). With 18 species, Neobisiidae is the most diverse family with species spanning two genera: Parobisium Chamberlin, 1930 and Bisetocreagris Ćurčić, 1983. Additionally, the families Chernetidae and Chthoniidae contain six and five species, respectively.

The pseudoscorpion genus Parobisium was first established by Chamberlin (1930) as a subgenus of Neobisium Chamberlin, 1930, and later elevated by Chamberlin and Malcolm (1960) to generic rank. Parobisium is characterized by the absence of a galea on the movable cheliceral finger, fixed chelal finger with a compact subterminal cluster of only three tactile setae (et, it, est), and a more diffuse subbasal to basal cluster of five tactile setae (isb, ist, ib, esb, eb) (Chamberlin 1962). However, for some North American and Asian Parobisium species, the trichobothrium (est) is isolated in the distal half of the fixed finger and has a trichobothrial pattern quite similar to Bisetocreagris.

The key character used to distinguish between these two genera is that Bisetocreagris usually has elongate galeae. Mahnert and Li (2016) intimated the galea is extremely fragile in Bisetocreagris species. This implies galea may be easily broken or damaged during collection or transport (Y. Li, pers. com., 18 December 2019). Subsequently, using galeae as a diagnostic character for describing and identifying species may hinder accurate classification of this group. In general, Parobisium differs from Bisetocreagris as there is a distinct and rounded sclerotic knob, rather than an absence of galea (Mori-kawa 1960; Hong 1996; Mahnert and Li 2016).

During the examination of Guizhou specimens collected by Mingyi Tian (of the South China Agricultural University, Guangzhou Province) between 2013 and 2017, we identified several species of Parobisium, which may be undescribed. In most cases, we had too few specimens to formally describe the species, and in some cases we had only one specimen. Unfortunately, this can be limiting in describing new species, especially given the aforementioned considerations with the galea. To address this problem, the lead author and colleagues collected additional specimens at the
Cave-dwelling pseudoscorpions of China

With additional specimens, we were able to both describe these species and confirm that these *Parobisium* species have a distinct and rounded sclerotic knob rather than the absence of galae.

Based upon specimens collected by both M. Tian and the lead author, we describe four new species of *Parobisium* from caves in Guizhou Province, China. All species are subterranean-adapted, and include *P. motianense* sp. nov., *P. qiangzhuang* sp. nov., *P. sanlouense* sp. nov., and *P. tiani* sp. nov. We also provide recommendations for management of these caves and the cave arthropod communities and habitats they support.

**Material and methods**

**Study area**

Guizhou, located in the Yunnan-Guizhou Plateau, is the centrally located province within the SCK. The karst escarpment within this area is approximately 130,000 km² encompassing 73% of Guizhou Province (Rong and Yang 2004; He and Li 2016). Karst formation in Guizhou emerged from a plate group from the Proterozoic to the Quaternary Period, and consists mainly of shallow marine carbonate deposits (Zhou et al. 2017). The extensive distribution of carbonate geology and the subtropical monsoon climate provide suitable conditions for the development of karst caves. According to Zhou et al. (2017), Guizhou supports at least 4,960 caves.

The lead author and colleagues searched for and collected pseudoscorpions within three of the four Guizhou caves (Fig. 1) where M. Tian initially collected specimens. As none of these caves were subject to previous studies or exploration efforts, cave maps were not available. We have provided estimations of entrance configuration and cave length, as well as information on surface vegetation and adjacent human activities.

Motian Cave (Figs 1C, 18) is located ~2 km southwest of Tangbian Town, Pingtang County. This limestone cave has one downward sloping oval entrance (~8 meters high by ~4 meters wide), approximately 2100 meters in length, and extends horizontally. The cave is surrounded by agriculture with the nearest rural residential area less than 100 m from the cave entrance.

Zharou Cave (Figs 1B, 19) is located ~1 km north of Daying Town, Ziyun County. This limestone cave has one triangular entrance (~2 meters high and ~3.5 meters wide), approximately 80 meters in length, and extends horizontally. The surrounding area is largely disturbed and characterized by low shrubs and weeds (Gramineae); agricultural fields and a rural residential area are approximately 100 m away.

Sanlou Cave (Figs 1D, 20) is located ~2.5 km northwest of Daoping Town, Fuquan City. This horizontally-trending cave is approximately 200 meters in length and has an irregularly round entrance (~4 meters in diameter). Situated near a sand mining operation, this cave is the primary water source for the village of Daoping. Subsequently, a reservoir and water delivery system was built in the deepest part of the cave. The cave has also been designated as a water source protection area.
Figure 1. Study area, general cave locations, and type locality for each species, Guizhou Province, China. A Biyun Cave, Parobisium tiani sp. nov. B Zharou Cave, Parobisium qiangzhuang sp. nov. C Motian Cave, Parobisium motianense sp. nov. D Sanlou Cave, Parobisium sanlouense sp. nov.

Biyun Cave (Figs 1A, 21) is located in Biyun Park, Chengguan Town, Pan County and is less than 50 m from a rural residential area. This cave has two entrances. One entrance is dome-shaped (~30 meters wide at the base and 10 m high); during the rainy season, a river flows into this cave entrance. The second entrance is located about 80 m uphill from the lower entrance and is irregularly round in shape (~30 meters in diameter). This cave was developed as a tourist cave, and has an unmaintained footpath paved with concrete, which connects the two entrances.

Field sampling

From 29 July to 5 August 2019, researchers conducted direct intuitive searches (sensu Wynne et al. 2019) in the estimated deep zone of each cave by examining bat guano, dead insects, edges of pools and streams, flood detritus, and mud floors (Figs 19B, 20C). Two observers spent approximately two hours searching in Zharou Cave, four hours searching in Biyun cave, and four observers spent about three hours searching Sanlou Cave.
Preparation and analysis

Specimens were preserved in 75% ethanol and deposited in the Museum of Hebei University (MHBU), Baoding, China. Photographs were taken using a Leica M205A stereomicroscope equipped with a Leica DFC550 camera and LAS software (Ver. 4.6). We used a Leica M205A stereomicroscope (with a drawing tube) for drawings and measurements. Chela and chelal hand were measured in ventral view. All measurements are in millimeters (mm) unless noted otherwise. Detailed examination of characters was done using an Olympus BX53 general optical microscope. Temporary slide mounts were prepared in glycerol.

Terminology

Cave ecosystems typically consist of four environmental zones (Howarth 1980, 1983): (1) entrance zone—or light zone, which represents a combination of surface and cave environmental conditions; (2) twilight zone—occurring slightly deeper within the cave and has both diminished light conditions and direct influence of surface environment; (3) transition zone—aphotic, yet barometric and diurnal shifts may still occur at a significantly diminished rate, but the climate is approaching near stable conditions; and, (4) deep zone—complete darkness, high environmental stability, near stable temperature, near water-saturated atmosphere, and low to no airflow (usually in the deepest part of the cave). The deep zone represents the region most conducive to supporting subterranean-adapted animals. Although there are four primary cave specific functional groups recognized, the species discussed here have been identified as either troglobionts or troglrophiles. Troglobionts are obligate cave dwellers that require the stable environmental conditions of the deep zone to complete their life cycle and exhibit morphological characteristics (i.e., troglomorphisms) indicative of subterranean adaptation (Sket 2008). Troglrophiles (or troglophilous organisms) lack troglomorphic characters yet occur facultatively within caves and complete their life cycles there, but also occur in similar cave-like surface habitats (Barr 1967, Howarth 1983).

Pseudoscorpion terminology and measurements mostly follow Chamberlin (1931) with some minor modifications to the terminology of the trichobothria (Harvey 1992) and chelicera (Judson 2007). The following abbreviations used for the trichobothria: \( b \) = basal; \( sb \) = sub-basal; \( st \) = sub-terminal; \( t \) = terminal; \( ib \) = interior basal; \( isb \) = interior sub-basal; \( ist \) = interior sub-terminal; \( it \) = interior terminal; \( eb \) = exterior basal; \( esb \) = exterior sub-basal; \( est \) = exterior sub-terminal; and, \( et \) = exterior terminal.
Table 1. The 29 known cave-dwelling pseudoscorpion species from China. ‘Category’ indicates the functional group in which each species belongs – either troglobiont or troglophile. The number of caves (#Caves) may be used to infer the level of endemism. Names of administrative provinces where each species is presently known is also provided.

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<th>Reference</th>
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Results

Family Neobisiidae Chamberlin, 1930
Subfamily Neobisiinae Chamberlin 1930

Genus Parobisium Chamberlin 1930


Type species. Neobisium (Parobisium) magnum Chamberlin, 1930, by original designation.
Key to *Parobisium* species of China

1  Carapace with eyes or eye spots .................................................................2
   – Carapace without eyes or eye spots ..........................................................5
2  Carapace only with two eyes or eye spots ................................. *P. tiani* sp. nov.
   – Carapace with four eyes or eyespots .......................................................3
3  Eight setae on posterior margin of carapace; pedipalpal femur 4.65 times longer than wide, patella 3.14 times longer than wide ................................................................. *P. xiaowutaicum* Guo & Zhang, 2016
   – Six setae on posterior margin of carapace; both pedipalpal femur and patella more than 5.7 times longer than wide .................................................................4
4  Pedipalp without granulation; pedipalpal femur 8.91–8.97 times longer than wide, patella 7.64–7.84 times longer than wide ................................................................. *P. magangensis* Feng, Wynne & Zhang, 2019
   – Pedipalp with granulation present on femur, inside lateral of patella and chelal hand; pedipalpal femur 6.75 times longer than wide, patella 5.7 times longer than wide ................................................................. *P. yuantongi* Feng, Wynne & Zhang, 2019
5  Carapace with four developed eyes; epistome small, triangular; pedipalp without granulation ................................................................. *P. wangae* Guo & Zhang, 2016
   – Carapace with four eyespots; epistome small, rounded; pedipalp with finely granulation ........................................................................................................6
6  Pedipalpal femur 6.50–6.59 times longer than wide, patella 5.07–5.11 times longer than wide ................................................................. *P. sanlouense* sp. nov.
   – Both pedipalpal femur and patella less than 5.0 times longer than wide ..........7
7  Femur of pedipalp with granulation; pedipalpal femur 3.89–4.11 times longer than wide, patella 2.54–2.60 times longer than wide .......................... *P. qiangzhuang* sp. nov.
   – Femur of pedipalp without granulation; pedipalpal femur 4.66–4.9 times longer than wide, patella 3.09–3.39 times longer than wide ........ *P. motianense* sp. nov.

*Parobisium motianense* sp. nov.

http://zoobank.org/D19923FF-CC27-4B00-9A9E-4DB225825490
Figs 2–5

**Type material.** Holotype male (Ps.-MHBU- GZ17051801): China, Guizhou Province, Pingtang County, Tangbian Town, Motian Cave (Figs 1C, 18), [25°38’32.86”N, 104°46’00.36”E], 869 m elevation, 18 May 2017, Mingyi Tian leg. Paratypes: 2 males (Ps.-MHBU- GZ17051802 & GZ170501803), 1 female (Ps.-MHBU- GZ17051804), same data as for holotype.

**Etymology.** Latinized adjective derived from the name of the type locality, Motian Cave.

**Distribution.** This species is known only from the type locality.

**Diagnosis.** Prior to this study, only four species of *Parobisium* have been reported in China (*Parobisium wangae* Guo & Zhang, 2016, *Parobisium xiaowutaicum* Guo &
Zhang, 2016, *Parobisium magangensis* Feng, Wynne & Zhang, 2019 and *Parobisium yuantongi* Feng, Wynne & Zhang, 2019). The new troglomorphic species can be distinguished from other members of the genus *Parobisium* by following combination of characters: carapace with four eye spots on a raised surface (*P. wangae* has four developed eyes, *P. tian* with two faint eye spots; *P. magangensis*, *P. xiaowutaicum* and *P. yuantongi* lacks eyes/eye spots); epistome small, rounded (small, triangular in *P. wangae*; triangular, with rounded top in *P. tian* and *P. yuantongi*); carapace with six setae on posterior margin (eight in *P. wangae*; eight in *P. xiaowutaicum*); pedipalpal femur 4.66–4.90 times longer than wide (8.91–8.97 times in *P. magangensis*; 3.89–4.11 times in *P. qiangzhuang*; 6.50–6.59 times in *P. sanlouense*; 5.63–5.73 times in *P. tian*; 3.60–3.65 times in *P. wangae*; 6.75 times in *P. yuantongi*); patella 3.09–3.39 times longer than wide (7.64–7.84 times in *P. magangensis*; 2.54–2.60 times in *P. qiangzhuang*; 5.07–5.11 times in *P. sanlouense*; 4.52–4.58 times in *P. tian*; 1.89–2.16 times in *P. wangae*; 5.70 times in *P. yuantongi*); pedipalpal hand which is finely granular (smooth in *P. magangensis*, *P. wangae* and *P. xiaowutaicum*; with granulation present on inside lateral of femur and chelal hand in *P. qiangzhuang* and *P. sanlouense*; with granulation present on femur, inside lateral of patella and chelal hand in *P. yuantongi*); chela (with pedicel) 3.72–4.06 times longer than wide (8.67–8.69 times in *P. magangensis*; 3.12–3.25 times in *P. qiangzhuang*; 6.08–6.34 times in *P. sanlouense*; 4.97–5.03 times in *P. tian*; 3.13–3.52 times in *P. wangae*; 3.14 times in *P. xiaowutaicum*; 5.70 times in *P. yuantongi*); both chelal finger has 95–98 teeth (146–162 in *P. magangensis*; 69–80 in
**Figure 3.** *Parobisium motianense* sp. nov. A Holotype male, dorsal view B Paratype female, dorsal view.

*P. qiangzhuang*; 119–130 in *P. sanlouensis*; 57–74 in *P. wangaen*; 73–75 in *P. xiaowutai.cum*; 116–118 in *P. yuantongi*).

**Description.** Male (Fig. 3A). Carapace, chelicerae, and pedipalps yellowish brown to reddish brown; abdomen and legs yellowish.

Carapace (Figs 4A, 5A): Smooth, 1.16–1.20 times longer than broad, with a total of 28–30 setae, including 4 on anterior margin, 6 on posterior margin, and 1 on each side of anterior lateral margin; with four eye spots on a raised surface (Fig. 4B); epistome small, rounded.

Chelicera (Figs 4C, 5B): Hand with 7 setae, movable finger with 1 submedial seta; fixed finger with 12–14 teeth; movable finger with 14–15 teeth; serrula exterior with 40–44 lamellae; serrula interior with 25–29 lamellae. Galea (Fig. 5E) replaced by a small rounded transparent sclerotic knob. Rallum (Fig. 5C) with 8 pinnate blade, distal-most blade with expanded base, and together with the second blade slightly separated from the others, proximal one short.

Pedipalps (Figs 4D–E, 5H–J): Apex of coxa rounded, with 5 setae on each side, pedipalpal coxa with 7 setae. Pedipalp smooth and slender except for hand, which is
finely granular. Trochanter 2.04–2.22 times longer than wide, femur 4.66–4.90, patella 3.09–3.39 times longer than wide, pedicel about half the entire length of patella, chela (with pedicel) 3.72–4.06, chela (without pedicel) 3.35–3.71 times longer than wide, movable finger 1.43–1.45 times longer than hand (without pedicel). Fixed chelal finger with 8 trichobothria, movable finger with 4, \( eb \) and \( esb \) on lateral margin of hand; \( ib, ist, \) and \( isb \) closely grouped at the base of the fixed finger; \( est \) slightly distal of finger middle; \( it \) closer to fingertip than \( er \); on movable finger, \( st \) nearer to \( t \) than to \( sb \), the distance between \( sb \) and \( b \) is somewhat equal to that of \( sb \) and \( st \) (Figs 4D, 5H–I). Venom apparatus present only in fixed chelal finger, venom duct short, not extending
Figure 5. Parobisium motianense sp. nov., holotype male (A–F, H–L), female (G). A Carapace, dorsal view B Left chelicera, dorsal view C Rallum D Subterminal tarsal seta E Movable finger of chelicera, showing sclerotic knob F Male genitalia G Female genitalia H Right chela, dorsal view I Right chelal fingers, lateral view J Right pedipalp, dorsal view (trochanter, femur, and patella) K Left leg I, lateral view L Left leg IV, lateral view. Scale bars: 0.1 mm (C, D–E), 0.25 mm (B, F–G), 0.5 mm (A, I), 1 mm (H, J–L).
beyond half the distance to et. Fixed chelal finger with 96–98 teeth, movable finger with 95–97 teeth.

XI): 9–10/14–16/15–16/15–13/16–12/14/12–15/2; stigmata with 5–6 setae; anal
cone with 2 dorsal and 2 ventral setae. Male genital area (Figs 4F, 5F): sternite II with
35–38 scattered setae; sternite III with anteromedian groove flanked by one small seta
on each side, with 20–26 posterior setae.

5.57 times longer than deep, femur 1.55–1.61 times longer than patella, telotarsus
1.54–1.56 times longer than basitarsus. Leg IV (Figs 4I, 5L): femur + patella 4.36–
5.00 times longer than deep, femur shorter than patella; tibia 8.04–9.05, basitarsus
3.81–3.88, telotarsus 5.44–6.36 times longer than deep, telotarsus 1.35–1.43 times
longer than basitarsus; tibia with one tactile setae (TS=0.52–0.54), basitarsus with
one tactile setae (TS=0.15–0.16), telotarsus with one tactile setae (TS=0.49–0.53);
subterminal tarsal seta (Fig. 5D) bifurcate; arolium not divided, shorter than the slen-
der and simple claws.

Female (paratype; Fig. 3B): Mostly same as holotype.

Chelicera. Hand with 7 setae, movable finger with 1 submedial seta; fixed finger
with 15–17 teeth; movable finger with 14–15 teeth; serrula exterior with 41 lamellae;
serrula interior with 23 lamellae. Galea replaced by a conspicuous semicircular trans-
parent sclerotized knob; rallum of 8 blades, similar to holotype.

Pedipalps. Pedipalpal coxa with 8–9 setae. Trochanter 2.08, femur 4.52, patella
3.02, chela (with pedicel) 3.69, chela (without pedicel) 3.33 times longer than wide,
movable finger 1.22 times longer than hand (without pedicel). Fixed chelal finger with
97 teeth, movable finger with 95 teeth.

Abdomen. Tergal chaetotaxy (I–XI): 8/7/9/10/11/11/11/11/10/6; sternal chaet-
otaxy (IV–XI): 11/16/17/16/16/14/12/2. Female genital area (Figs 4G, 5G): sternite II
with 6–7 setae on each side; sternite III with a row of 20 setae on the posterior margin.

Measurements: (length/breadth or depth in mm; ratios for most characters in pa-
rentheses). Male (holotype and paratypes). Body length 4.26–4.78. Carapace 1.16–
1.20 (1.31–1.36/1.13). Pedipalpal trochanter 2.04–2.22 (0.91–0.96/0.41–0.47),
femur 4.66–4.90 (1.96–2.05/0.40–0.44), patella 3.09–3.39 (1.66–1.73/0.49–0.56),
chela (with pedicel) 3.72–4.06 (3.09–3.13/0.77–0.83), chela (without pedicel)
3.35–3.71 (2.78–2.86/0.77–0.83), hand length (without pedicel) 1.30–1.31, mov-
able finger length 1.86–1.90 (1.43–1.45 times longer than hand without pedicel). Leg
I: trochanter 1.36–1.50 (0.39/0.26–0.28), femur 4.37–4.96 (1.18–1.19/0.24–0.27),
patella 2.92–3.22 (0.74–0.76/0.23–0.26), tibia 6.00–7.27 (1.02–1.09/0.15–0.17),
basitarsus 3.33–3.85 (0.50/0.13–0.15), telotarsus 5.57–5.50 (0.77–0.78/0.14). Leg
IV: trochanter 2.13–2.44 (0.64–0.67/0.27–0.30), femur + patella 4.36–5.00 (1.90–
1.92/0.38–0.44), tibia 8.04–9.05 (1.85–1.90/0.21–0.23), basitarsus 3.81–3.88
(0.61–0.66/0.16–17), telotarsus 5.44–6.36 (0.87–0.89/0.14–0.16).
Female (paratype). Body length 5.99. Carapace 1.14 (1.46/1.28). Pedipalpal trochanter 2.08 (1.02/0.49), femur 4.52 (2.08/0.46), patella 3.02 (1.81/0.60), chela (with pedicel) 3.69 (3.28/0.89), chela (without pedicel) 3.33 (2.96/0.89), hand length (without pedicel) 1.47, movable finger length 1.79 (1.22 times longer than hand without pedicel). Leg I: trochanter 1.47 (0.44/0.30), femur 4.62 (1.20/0.26), patella 3.57 (0.82/0.23), tibia 6.76 (1.15/0.17), basitarsus 3.40 (0.51/0.15), telotarsus 5.13 (0.77/0.15). Leg IV: trochanter 2.28 (0.73/0.32), femur + patella 5.10 (1.99/0.39), tibia 8.58 (2.06/0.24), basitarsus 3.72 (0.67/0.18), telotarsus 5.29 (0.90/0.17).

**Parobisium qiangzhuang** sp. nov.
http://zoobank.org/F122663A-2AF0-437C-83C5-791E9739854C

Figs 6–9

**Type material.** Holotype male (Ps.-MHBU- GZ19080301): China, Guizhou Province, Anshun City, Ziyun County, Daying Town, Zharou Cave (Figs 1B, 19), [25°29'24.87"N, 106°18'28.65"E], estimated cave deep zone, 1139 m elevation, 3 August 2019, Zegang Feng, Chen Zhang leg. Paratypes: 2 Males (Ps.-MHBU-GZ19080302- GZ19080303), same data as for holotype; 1 Female (Ps.-MHBU-GZ19061201), same location as holotype, 12 June 2018, Sunbin Huang, Zhuanghui Qin, Mengzhen Chen, Lei Tao leg.

**Etymology.** The species name, *qiangzhuang*, was derived from the Latinized Mandarin phrase for “strong and hardy” qiáng zhàng (强壮), which refers to the shape of chela.

**Distribution.** Species known only from the type locality.

**Diagnosis.** The subterranean-adapted *Parobisium qiangzhuang* can be distinguished from other members of the genus *Parobisium* by following combination of characters: carapace with four eye spots on a raised surface (*P. wangae* has four developed eyes, *P. tiani* with two faint eye spots; *P. magangensis*, *P. xiaowutaicum* and *P. yuantongi* lacks eyes/eye spots); epistome small, rounded (small, triangular in *P. wangae*; triangular, with rounded top in *P. tiani* and *P. yuantongi*); pedipalpal femur 3.89–4.11 times longer than wide (8.91–8.97 times in *P. magangensis*; 4.66–4.90 times in *P. motianense*; 6.50–6.59 times in *P. sanlouense*; 5.63–5.73 times in *P. tiani*; 4.65 times in *P. xiaowutaicum*; 6.75 times in *P. yuantongi*); patella 2.54–2.60 times longer than wide (7.64–7.84 times in *P. magangensis*; 3.09–3.39 times in *P. motianense*; 5.07–5.11 times in *P. sanlouense*; 4.52–4.58 times in *P. tiani*; 3.14 times in *P. xiaowutaicum*; 5.70 times in *P. yuantongi*); pedipalpal hand and inside lateral of femur, which is finely granular (smooth in *P. magangensis*, *P. wangae* and *P. xiaowutaicum*; with granulation present on chelal hand in *P. tiani*; with granulation present on femur, inside lateral of patella and chelal hand in *P. yuantongi*); chela (with pedicel) 3.12–3.52 times longer than wide (8.67–8.69 times in *P. magangensis*; 3.72–4.06 times in *P. motianense*; 6.08–6.34 times in *P. qiangzhuang*; 4.97–5.03 times in *P. tiani*; 5.70 times in *P. yuantongi*); both chelal finger has 69–80 teeth (146–162 in *P. magangensis*; 96–98 in *P. motianense*; 119–130 in *P. sanlouensis*; 104–112 in *P. tiani*; 116–118 in *P. yuantongi*).
Figure 6. *Parobisium qiangzhuang* sp. nov. Male habitus.

**Description.** Male (Fig. 7A). Carapace, chelicerae, and pedipalps reddish brown; abdomen and legs yellowish.

Carapace (Figs 8A, 9A): Smooth, 1.15–1.24 times longer than broad, with a total of 28–31 setae, including 4 on anterior margin, 6–8 on posterior margin, and 1–2 on each side of anterior lateral margin; with 4 eye spots on a raised surface (Fig. 8B); epistome small and rounded.

Chelicera (Figs 8C, 9B): Hand with 7 setae, movable finger with 1 submedial seta; fixed finger with 9–11 teeth; movable finger with 10–13 teeth; serrula exterior with 34–38 lamellae; serrula interior with 20–24 lamellae. Galea (Fig. 9D) replaced by a small rounded transparent sclerotic knob. Rallum (Fig. 9C) with 8 pinnate blade, distal-most blade with expanded base, proximal one short.

Pedipalps (Figs 8D–E, 9H–J): Apex of coxa rounded, with 5 setae on each side, pedipalpal coxa with 9 setae. Pedipalp smooth except for hand and inside lateral of femur, which is finely granular. Trochanter 1.84–1.97 times longer than wide, femur 3.89–4.11, patella 2.54–2.60 times longer than wide, pedicel about half the entire length of patella, chela (with pedicel) 3.12–3.25, chela (without pedicel) 2.86–2.97 times longer than wide, movable finger 1.11–1.15 times longer than hand (without pedicel). Fixed chelal finger with 8 trichobothria, movable finger with 4, *eb* and *esb* on lateral margin of hand; *ib*, *ist* and *isb* closely grouped at the base of the fixed finger; *est* slightly distal of finger middle; *it-et* at same level near fingertip; on movable finger *st* nearer to *t* than to *sb*, the distance between *sb* and *b* is somewhat equal to that of *sb* and *st* (Figs 8D, 9H–I). Venom apparatus present only in fixed chelal finger, venom
duct short, not extending past half of the distance to et. Fixed chelal finger with 71–75 teeth, movable finger with 69–80 teeth.

Abdomen: Pleural membrane granulated. Tergal chaetotaxy (I–XI): 10–11/11–12/12/12–13/12/12/12/12–13/12/7–9; sternal chaetotaxy (IV–XI): 9–11/13–15/14–16/13–16/13–15/12–14/12–15/3–4; stigmata with 4–5 setae; anal cone with 2 dorsal and 2 ventral setae. Male genital area (Figs 8F, 9E): sternite II with 28–30 scattered setae; sternite III with anteromedian groove flanked by one small seta on each side, with 17–19 posterior setae.

Legs: Coxa chaetotaxy (I–IV): 9–11/7–10/4–5/9–11. Leg I (Figs 8H, 9K): femur 3.52–3.55, patella 2.89–2.94, tibia 5.85–6.67, basitarsus 2.67–2.82, telotarsus 4.42–4.82 times longer than deep, femur 1.40–4.42 times longer than patella, telotarsus 1.66–1.71 times longer than basitarsus. Leg IV (Figs 8I, 9L): femur + patella 3.63–3.65 times longer than deep, femur shorter than patella, tibia 6.45–6.79, basitarsus 2.93, telotarsus 4.40–4.57 times longer than deep, telotarsus 1.50–1.56 times longer than basitarsus; tibia with one tactile setae (TS=0.48–0.50), basitarsus with one tactile setae (TS=0.11–0.15), telotarsus with one tactile setae (TS=0.41–0.45); subterminal tarsal seta (Fig. 9G) bifurcate, both branches dentate; arolium not divided, shorter than the slender and simple claws.

Female (paratype) (Fig. 7B): Mostly same as holotype.

Chelicera. Hand with 7 setae, movable finger with 1 submedial seta; fixed finger with 13 teeth; movable finger with 12 teeth; serrula exterior with 38 lamellae; serrula interior with 24 lamellae. Galea replaced by conspicuous semicircular transparent sclerotic knob; rallum of 8 blades, but similar to holotype.

Pedipalps. Pedipalpal coxa with 10–11 setae. Trochanter 2.02, femur 3.60, patella 2.18, chela (with pedicel) 2.86, chela (without pedicel) 2.63 times longer than wide, movable finger 1.01 times longer than hand (without pedicel). Fixed chelal finger with about 72 teeth, movable finger with about 78 teeth.
Figure 8. *Parobisium qiangzhuang* sp. nov. holotype male (A–F, H–I), female (G): A Carapace, dorsal view B Eye area, lateral view C Right chelicera, dorsal view D Right chela, lateral view E Right pedipalp, dorsal view F Male genitalia G Female genitalia H Right leg I, lateral view I Right leg IV, lateral view.
Figure 9. *Parobisium qiangzhuang* sp. nov., holotype male (A–E, G–L), female (F). A Carapace, dorsal view B Right chelicera, dorsal view C Rallum D Movable finger of chelicera, showing sclerotic knob E Male genitalia F Female genitalia G Subterminal tarsal seta H Right chela, dorsal view I Right chelal fingers, lateral view J Right pedipalp, dorsal view (trochanter, femur, and patella) K Right leg I, lateral view L Right leg IV, lateral view. Scale bars: 0.1 mm (C–D, G), 0.25 mm (B, E–F), 0.5 mm (A, K–L), 1 mm (H–J).
Abdomen. Tergal chaetotaxy (I–XI): 8/11/13/13/12/12/13/13/13/12/5; sternal chaetotaxy (IV–XI): 7/16/14/14/15/14/12/5. Female genital area (Figs 8G, 9F): sternite II with 4 setae on each side; sternite III with a row of 12 setae on the posterior margin.

Measurements: (length/breadth or depth in mm; ratios for most characters in parentheses). Male (holotype and paratypes). Body length 3.61–4.42. Carapace 1.15–1.24 (1.12–1.18/0.95–0.97). Pedipalpal trochanter 1.84–1.97 (0.70–0.75/0.38), femur 3.89–4.11 (1.40–1.48/0.36), patella 2.54–2.60 (1.22–1.30/0.48–0.50), chela (with pedicel) 3.12–3.25 (2.18–2.28/0.67–0.73), chela (without pedicel) 2.86–2.97 (1.99–2.09/0.67–0.73), hand length (without pedicel) 1.02–1.11, movable finger length 1.17–1.23 (1.11–1.15 times longer than hand without pedicel). Leg I: trochanter 1.17–1.30 (0.27–0.30/0.23), femur 3.52–3.55 (0.74–0.78/0.21–0.22), patella 2.89–2.94 (0.53–0.55/0.18–0.19), tibia 5.85–6.67 (0.76–0.80/0.12–0.13), basitarsus 2.67–2.82 (0.31–0.32/0.11–0.12), telotarsus 4.42–4.82 (0.53/0.11–0.12). Leg IV: trochanter 2.08–2.30 (0.50–0.53/0.23–0.24), femur + patella 3.63–3.65 (1.24–1.27/0.34–0.35), tibia 2.93 (0.41–0.44/0.14–0.15), basitarsus 3.81–3.88 (0.61–0.66/0.16–17), telotarsus 4.40–4.57 (0.64–0.66/0.14–0.15).

Female (paratype). Body length 5.49. Carapace 1.24 (1.50/1.21). Pedipalpal trochanter 2.02 (0.91/0.45), femur 3.60 (1.69/0.47), patella 2.18 (1.46/0.67), chela (without pedicel) 2.86 (2.69/0.94), chela (without pedicel) 2.63 (2.47/0.94), hand length (without pedicel) 1.37, movable finger length 1.38 (1.01 times longer than hand without pedicel). Leg I: trochanter 1.26 (0.34/0.27), femur 3.54 (0.85/0.24), patella 2.86 (0.63/0.22), tibia 7.08 (0.92/0.13), basitarsus 3.17 (0.38/0.12), telotarsus 4.46 (0.58/0.13). Leg IV: trochanter 2.07 (0.60/0.29), femur + patella 4.11 (1.56/0.38), tibia 7.55 (1.51/0.20), basitarsus 2.81 (0.45/0.16), telotarsus 4.67 (0.70/0.15).

### Parobisium sanlouense sp. nov.
http://zoobank.org/EC06FD6A-41EB-47A5-A31A-BC2EC89941A7
Figs 10–13

**Type material.** Holotype male (Ps.-MHBU-GZ15050201): China, Guizhou Province, Fuquan County, Sanlou Cave (Figs 1D, 20), [26°56’46”N, 107°18’47”E], 1280 m elevation, 02 May 2015, Mingyi Tian leg. Paratypes: 3 males (Ps.-MHBU-GZ19072901, GZ19072902, GZ19072903), 3 females (Ps.-MHBU-GZ19072904, GZ19072905, GZ19072906), same location as holotype, estimated cave deep zone, 29 July 2019, Zegang Feng, Chen Zhang, Zhaoyi Li, Yonghao Li leg.

**Etymology.** Latinized adjective derived from the name of the type locality, Sanlou Cave.

**Distribution.** This species is known only from the type locality.

**Diagnosis.** This new species can be easily distinguished from other members of the genus *Parobisium* by following combination of characters: carapace with four eye spots on a slightly raised (*P. wangae* has four developed eyes, *P. tiani* with two faint eye spots; *P. magangensis, P. xiaowutaicum* and *P. yuantongi* lacks eyes/eye spots); epistome small, rounded (small, triangular in *P. wangae*; triangular, with rounded top in *P. tiani* and *P. yuantongi*); pedipalpal femur 6.50–6.59 times longer than wide (8.91–8.97 times in
P. magangensis; 4.66–4.90 times in *P. motianense*; 3.89–4.11 times in *P. qiangzhuang*; 5.63–5.73 times in *P. tiani*; 3.60–3.65 times in *P. wangae*; 4.65 times in *P. xiaowutaiicum*); patella 5.07–5.11 times longer than wide (7.64–7.84 times in *P. magangensis*; 3.09–3.39 times in *P. motianense*; 2.54–2.60 times in *P. qiangzhuang*; 4.52–4.58 times in *P. tiani*; 1.89–2.16 times in *P. wangae*; 3.14 times in *P. xiaowutaiicum*; 5.70 times in *P. yuantongi*); pedipalpal hand and inside lateral of femur, which is finely granular (smooth in *P. magangensis*, *P. wangae* and *P. xiaowutaiicum*; with granulation present on chelal hand in *P. tiani*; with granulation present on femur, inside lateral of patella and chelal hand in *P. yuantongi*); chela (with pedicel) 6.08–6.34 times longer than wide (8.67–8.69 times in *P. magangensis*; 3.72–4.06 times in *P. motianense*; 3.12–3.25 times in *P. qiangzhuang*; 4.97–5.03 times in *P. tiani*; 3.13–3.52 times in *P. wangae*; 3.14 times in *P. xiaowutaiicum*; 5.70 times in *P. yuantongi*); both chelal finger has 119–130 teeth (146–162 in *P. magangensis*; 95–98 in *P. motianense*; 69–80 in *P. qiangzhuang*; 57–74 in *P. wangae*; 73–75 in *P. xiaowutaiicum*).

**Description.** Male (Fig. 11A). Carapace, chelicerae, and pedipalps reddish brown or yellowish brown; abdomen and legs yellowish.

Carapace (Figs 12A, 13A): Smooth, 1.21–1.30 times longer than broad, with a total of 29–31 setae, including 4 on anterior margin, 7 on posterior margin, and 1–2 on each side of anterior lateral margin; with 4 eye spots on a slightly raised surface (Fig. 12B); epistome small, rounded.

Chelicera (Figs 12C, 13B): Hand with 7 setae, movable finger with 1 submedial seta; fixed finger with 13–14 teeth; movable finger with 13–15 teeth; serrula exterior with 39–40 lamellae; serrula interior with 25–27 lamellae. Galea (Fig. 13F) replaced...
by a small rounded transparent sclerotic knob. Rallum (Fig. 13C) with 8 pinnate blade, distal-most blade with expanded base, proximal one short.

Pedipalps (Figs 12D–E, 13H, J–K): Apex of coxa rounded, with 5 setae on each side, pedipalpal coxa with 8–10 setae. Pedipalp smooth and slender except for hand and inside lateral of femur, which is finely granular. Trochanter 2.86–2.92 times longer than wide, femur 6.50–6.59, patella 5.07–5.11 times longer than wide, pedicel about half the entire length of patella, chela (with pedicel) 6.08–6.34, chela (without pedicel) 5.44–5.62 times longer than wide, movable finger 1.51–1.57 times longer than hand (without pedicel). Fixed chelal finger with 8 trichobothria, movable finger with 4, \( eb \) and \( esb \) on lateral margin of hand; \( ib \), \( ist \) and \( isb \) closely grouped at the base of the fixed finger; \( est \) slightly distal of finger at middle; \( it \) slightly closer to fingertip than \( et \); on movable finger \( st \) nearer to \( t \) than to \( sb \), the latter slightly nearer \( b \) than to \( st \) (Figs 12D, 13H, J). Venom apparatus present only in fixed chelal finger, venom duct short, not extending past half of the distance to \( et \). Fixed chelal finger with 126–130 teeth, movable finger with 119–126 teeth.


Figure 12. Parobisium sanlouense sp. nov., holotype male (A–F, H–I), female (G). A Carapace, dorsal view B Eye area, lateral view C Right chelicera, dorsal view D Right chela, lateral view E Right pedipalp, dorsal view F Male genitalia G Female genitalia H Right leg I, lateral view I Right leg IV, lateral view.
1.52 times longer than basitarsus. Leg IV (Figs 12I, 13M): femur + patella 5.22–5.97 times longer than deep, femur shorter than patella, tibia 8.95–9.10, basitarsus 4.06–4.19, telotarsus 6.93–6.86 times longer than deep, telotarsus 1.43–1.49 times longer basitarsus; basitarsus with one tactile setae (TS=0.12–0.13), telotarsus with one tactile setae (TS=0.43–0.52); subterminal tarsal seta (Fig. 13I) bifurcate, both branches dentate; arolium not divided, shorter than the slender and simple claws.

Female (paratypes) (Fig. 11B): Mostly same as holotype.

Chelicera. Hand with 7 setae, movable finger with 1 submedial seta; fixed finger with 13–16 teeth; movable finger with 14–15 teeth; serrula exterior with 37–41 lamellae; serrula interior with 23–27 lamellae. Galea (Fig. 13G) replaced by conspicuous semicircular transparent sclerotic knob; rallum of 8–10 blades, similar to that of holotype.


Measurements: (length/breadth or depth in mm; ratios for most characters in parentheses). Male (holotype and paratypes). Body length 4.00–4.79. Carapace 1.21–1.30 (1.32–1.42/1.09–1.09). Pedipalpal trochanter 2.86–2.92 (1.03–1.08/0.36–0.37), femur 6.50–6.59 (2.34–2.44/0.36–0.37), patella 5.07–5.11 (2.23–2.25/0.44), chela (with pedicel) 6.08–6.34 (3.59–3.68/0.58–0.59), chela (without pedicel) 5.44–5.62 (3.21–3.26/0.58–0.59), hand length (without pedicel) 1.34–1.38, movable finger length 2.08–2.11 (1.51–1.57 times longer than hand without pedicel). Leg I: trochanter 1.54–1.57 (0.43–0.44/0.28), femur 5.08–5.73 (1.22–1.26/0.22–1.24), patella 3.82–4.05 (0.84–0.85/0.21–0.22), tibia 8.21–8.57 (1.15–1.20/0.14), basitarsus 4.00–4.33 (0.52/0.12–0.13), telotarsus 6.08–6.58 (0.73–0.79/0.12). Leg IV: trochanter 2.41–2.55 (0.70–0.74/0.29), femur + patella 5.22–5.97 (1.93–2.09/0.35–0.37), tibia 8.95–9.10 (1.88–1.91/0.21), basitarsus 4.06–4.19 (0.65–0.67/0.16), telotarsus 6.93–6.86 (0.96–0.97/0.14).

Female (paratypes). Body length 4.94–6.00. Carapace 1.36–1.39 (1.51–1.54/1.11). Pedipalpal trochanter 2.59–2.89 (1.01–1.07/0.37–0.39), femur 6.03–6.60 (2.17–2.31/0.36–0.35), patella 4.52–4.72 (1.99–2.17/0.44–0.46), chela (with pedicel) 5.07–5.35 (3.40–3.48/0.65–0.67), chela (without pedicel) 4.52–4.77 (3.03–3.10/0.65–0.67), hand length (without pedicel) 1.31–1.34, movable finger length 1.87–2.00 (1.40–1.53 times longer than hand without pedicel). Leg I: trochanter 1.48–1.56 (0.40–0.42/0.27), femur 5.09–5.48 (1.12–1.26/0.22–0.23), patella 3.90–4.05 (0.78–0.81/0.20), tibia 7.20–8.14 (1.08–1.14/0.14–0.15), basitarsus 3.69–4.00 (0.48–0.52/0.13), telotarsus 5.21–6.08 (0.73–0.79/0.13–0.14). Leg IV: trochanter 2.10–2.64 (0.61–0.74/0.28–0.29), femur + patella 4.87–5.72 (1.90–2.06/0.36–0.39), tibia 8.48–9.05 (1.78–1.99/0.21–0.22), basitarsus 3.88–4.19 (0.66–0.67/0.16–0.17), telotarsus 6.50–7.07 (0.91–0.99/0.14).
Figure 13. *Parobisium sanlouense* sp. nov., holotype male (A–D, G–M), female (E–F). A Carapace, dorsal view B Right chelicera, dorsal view C Rallum D Male genitalia E Female genitalia F Movable finger of chelicera (male), showing sclerotic knob G Movable finger of chelicera (female), showing sclerotic knob H Right pedipalp, dorsal view (trochanter, femur, and patella) I Subterminal tarsal seta J Right chelal fingers, lateral view K Right chela, dorsal view L Right leg I, lateral view M Right leg IV, lateral view. Scale bars: 0.5 mm (A–B), 0.1 mm (C, I), 0.25 mm (D–G), 1 mm (H, J–M).
Parobisium tiani sp. nov.
http://zoobank.org/5E7363D4-BF81-401D-94A2-EA7D654FFBC1
Figs 14–17

Type material. Holotype male (Ps.-MHBU-GZ13070901): China, Guizhou Province, Liupanshui City, Pan County, Chengguan Town, Biyun Cave (Figs 1A, 21), [25°46'29.97"N, 104°38'15.81"E], 1500 m elevation, 9 July 2013, Mingyi Tian leg. Paratypes: 1 female (Ps.-MHBU-GZ13070902), same location as holotype, 09 July 2013, Mingyi Tian leg; 2 males (Ps.-MHBU-GZ19080501, GZ19080502), 2 females (Ps.-MHBU-GZ19080503, GZ19080504) same location as holotype, estimated cave deep zone, 05 August 2019, Zegang Feng, Chen Zhang leg.

Etymology. The name is a patronym to honor Chinese cave biologist, Mingyi Tian. He provided us with his pseudoscorpion specimens and assisted in developing this study.

Distribution. This species is known only from the type locality.

Diagnosis. This new troglomorphic species can be easily distinguished from other members of the genus Parobisium by following combination of characters: carapace with two faint eye spots (P. wangae has four developed eyes, P. motianense, P. qiangzhuang and P. sanlouense with four eye spots; P. magangensis, P. xiaowutaicum and P. yuantongi lacks eyes/eye spots); epistome triangular, with rounded top (small, rounded in P. motianense, P. qiangzhuang and P. sanlouense; triangular, with rounded top in P. tiani and P. yuantongi; rounded in P. magangensis and P. xiaowutaicum); pedipalpal femur 5.63–5.75 times longer than wide (8.91–8.97 times in P. magangensis; 4.66–4.90 times in P. motianense; 3.89–4.11 times in P. qiangzhuang; 6.50–6.59 times in P. sanlouense; 3.60–3.65 times in P. wangae; 4.65 times in P. xiaowutaicum; 6.75 times in P. yuantongi); patella 4.52–4.58 times longer than wide (7.64–7.84 times in P. magangensis; 3.09–3.39 times in P. motianense; 2.54–2.60 times in P. qiangzhuang; 5.07–5.11 times in P. sanlouense; 1.89–2.16 times in P. wangae; 3.14 times in P. xiaowutaicum; 5.70 times in P. yuantongi); pedipalpal hand with granulation (smooth in P. magangensis, P. wangae and P. xiaowutaicum; with granulation present on chelal hand in P. tiani; with granulation present on inside lateral of femur and chelal hand in P. qiangzhuang and P. sanlouense; with granulation present on femur, inside lateral of patella and chelal hand in P. yuantongi); chela (with pedicel) 4.97–5.03 times longer than wide (8.67–8.69 times in P. magangensis; 3.72–4.06 times in P. motianense; 3.12–3.25 times in P. qiangzhuang; 6.08–6.34 times in P. sanlouense; 3.13–3.52 times in P. wangae; 3.14 times in P. xiaowutaicum; 5.70 times in P. yuantongi); both chelal finger has 104–112 teeth (146–162 in P. magangensis; 71–75 in P. qiangzhuang; 57–74 in P. wangae; 73–75 in P. xiaowutaicum).

Description. Male (Fig. 15A). Carapace, chelicerae, and pedpalps reddish brown or yellowish brown; abdomen and legs yellowish or yellowish brown.

Carapace (Figs 16A, 17A): Smooth, 1.22–1.27 times longer than broad, with a total of 24 setae, including 4 on anterior margin, 6 on posterior margin, and 1 on each side of anterior lateral margin; with 2 faint eye spots on a flat surface; epistome triangular, with rounded top.
Figure 14. <i>Parobisium tianii</i> sp. nov. Male habitus.

Chelicera (Figs 16C, 17B): Hand with 7 setae, movable finger with 1 submedial seta; fixed finger with 13–16 teeth; movable finger with 13–16 teeth; serrula exterior with 39–42 lamellae; serrula interior with 20–24 lamellae. Galea (Fig. 17D) replaced by a small rounded transparent sclerotic knob. Rallum (Fig. 17C) with 8 pinnate blade, distal-most blade with expanded base, and together with the second blade separated from the others, proximal one short.

Pedipalps (Figs 16E–F, 17H–I, K): Apex of coxa rounded, with 5 setae on each side, pedipalpal coxa with 7–8 setae. Pedipalp smooth and slender except for hand, which is finely granular. Trochanter 2.67–2.70 times longer than wide, femur 5.63–5.73, patella 4.52–4.58 times longer than wide, pedicel about half the entire length of patella, chela (with pedicel) 4.97–5.03, chela (without pedicel) 4.39–4.40 times longer than wide, movable finger 1.44–1.45 times longer than hand (without pedicel). Fixed chelal finger with 8 trichobothria, movable finger with 4, <i>eb</i> and <i>esb</i> on lateral margin of hand; <i>ib</i>, <i>ist</i> and <i>isb</i> closely grouped at the base of the fixed finger; <i>est</i> slightly distal of finger middle; <i>it</i> closer to fingertip than <i>et</i>; on movable finger, <i>st</i> nearer to <i>t</i> than to <i>sb</i>, the latter slightly nearer <i>st</i> than to <i>b</i> (Figs 16F, 17H–I). Venom apparatus present only in fixed chelal finger, venom duct short, not extending past half of the distance to <i>et</i>. Fixed chelal finger with 104–109 teeth, movable finger with 105–112 teeth.

Figure 15. *Parobisium tiani* sp. nov. A Holotype male, dorsal view B Paratype female, dorsal view.

34–43 scattered setae; sternite III with anteromedian groove flanked by one small seta on each side, with 15 posterior setae.

Legs: Coxa chaetotaxy (I–IV): 6–7/ 4–5/ 4–5/ 7. Leg I (Figs 16I, 17L): femur 6.27–6.50, patella 3.54–4.23, tibia 8.87–9.08, basitarsus 3.73–3.93, telotarsus 5.07–5.85 times longer than deep, femur 1.54–1.62 times longer than patella, telotarsus 1.36–1.38 times longer than basitarsus. Leg IV (Figs 16J, 17M): femur + patella 5.30–5.46 times longer than deep, femur shorter than patella, tibia 9.04–9.75, basitarsus 4.06–4.41, telotarsus 5.61–6.20 times longer than deep, telotarsus 1.35 times longer than basitarsus; basitarsus with a tactile setae in basally (TS=0.12–0.15), telotarsus with a tactile setae in middle (TS=0.46–0.47); subterminal tarsal seta (Fig. 17J) bifurcate, both branches dentate; arolium not divided, shorter than the slender and simple claws.

Female (paratypes) (Fig. 15B): Mostly same as holotype.

Chelicera. Hand with 7 setae, movable finger with 1 submedial seta; fixed finger with 13–16 teeth; movable finger with 12–19 teeth; serrula exterior with 39–41 lamellae; serrula interior with 22–23 lamellae. Galea (Fig. 17E) replaced by a semicircular transparent sclerotic knob; rallum of 8–9 blades, similar to holotype.

Pedipalps. Trochanter 2.44–2.61, femur 5.59–5.61, patella 4.38–4.57 times longer than wide; chela (with pedicel) 4.25–4.46 times longer than wide, chela (without pedicel) 3.79–4.00 times longer than wide, movable finger 1.26–1.28 times longer than hand (without pedicel). Fixed chelal finger with 96–106 teeth, movable finger with 97–105 teeth.

Figure 16. Parobisium tiani sp. nov., holotype male (A–C, E–G, I–J), female (D, H). A Carapace, dorsal view B Eye area, lateral view C Right chelicera of male, dorsal view D Right chelicera of female, dorsal view E Right pedipalp, dorsal view F Right chela, lateral view G Male genitalia H Female genitalia I Right leg I, lateral view J Right leg IV, lateral view.

15/12–13/12–14/3. Female genital area (Figs 16H, 17G): sternite II with 3–8 setae on each side; sternite III with a row of 13–16 setae on the posterior margin.
Figure 17. Parobisium tiani sp. nov., holotype male (A–D, F–M), female (E, G). A Carapace, dorsal view B Right chelicera, dorsal view C Rallum D Movable finger of chelicera (male), showing sclerotic knob E Movable finger of chelicera (female), showing sclerotic knob F Male genitalia G Female genitalia H Right chela, dorsal view I Right chelal fingers, lateral view J Subterminal tarsal seta K Right pedipalp, dorsal view (trochanter, femur, and patella) L Right leg I, lateral view M Right leg IV, lateral view. Scale bars: 0.1 mm (C–E, J), 0.25 mm (B, F–G), 0.5 mm (A), 1 mm (H–I, K–M).
Measurements: (length/breadth or depth in mm; ratios for most characters in parentheses). Male (holotype and paratypes). Body length 3.87–5.09. Carapace 1.22–1.27 (1.32–1.43/1.08–1.13). Pedipalpal trochanter 2.67–2.70 (1.04–1.16/0.39–0.43), femur 5.63–5.73 (2.29–2.42/0.40–0.43), patella 4.52–4.58 (2.17–2.38/0.48–0.52), chela (with pedicel) 4.97–5.03 (3.28–3.52/0.66–0.70), chela (without pedicel) 4.39–4.40 (2.90–3.08/0.66–0.70), hand length (without pedicel) 1.32–1.36, movable finger length 1.91–1.96 (1.44–1.45 times longer than hand without pedicel). Leg I: trochanter 1.35–1.50 (0.42/0.28–0.31), femur 6.27–6.50 (1.38–1.43/0.22), patella 3.54–4.23 (0.85–0.93/0.22–0.24), tibia 8.87–9.08 (1.18–1.33/0.13–0.15), basitarsus 3.73–3.93 (0.55–0.56/0.14–0.15), telotarsus 5.07–5.85 (0.76/0.13–0.15). Leg IV: trochanter 2.43–2.79 (0.68–0.78/0.28), femur + patella 5.30–5.46 (2.13–2.28/0.39–0.43), tibia 9.04–9.75 (2.17–2.34/0.24), basitarsus 4.06–4.41 (0.69–0.75/0.17), telotarsus 5.61–6.20 (0.93–1.01/0.15–0.18).

Female (paratypes). Body length 4.63–5.48. Carapace 1.19–1.27 (1.30–1.60/1.09–1.26). Pedipalpal trochanter 2.44–2.61 (0.95–1.15/0.39–0.44), femur 5.59–5.61 (2.18–2.47/0.39–0.44), patella 4.38–4.57 (2.06–2.47/0.47–0.54), chela (with pedicel) 4.25–4.46 (3.03–3.61/0.68–0.85), chela (without pedicel) 3.79–4.00 (2.72–3.22/0.68–0.85), hand length (without pedicel) 1.29–1.55, movable finger length 1.65–1.95 (1.26–1.28 times longer than hand without pedicel). Leg I: trochanter 1.47–1.60 (0.40–0.44/0.25–0.30), femur 5.39–5.92 (1.24–1.48/0.23–0.25), patella 3.75–4.18 (0.75–0.92/0.20–0.22), tibia 7.00–7.33 (1.05–1.32/0.15–0.18), basitarsus 3.14–4.07 (0.44–0.61/0.14–0.15), telotarsus 5.00–5.43 (0.65–0.76/0.13–0.14). Leg IV: trochanter 2.48–2.61 (0.67–0.81/0.27–0.31), femur + patella 5.91–5.97 (2.01–2.27/0.34–0.38), tibia 9.38–9.67 (1.97–2.32/0.21–0.24), basitarsus 3.82–4.53 (0.65–0.77/0.17), telotarsus 6.00–6.13 (0.90–0.98/0.15–0.16).

Discussion

Our work has increased the number of Chinese cave-dwelling pseudoscorpions from 25 to 29 species. In addition, we found that female individuals may have a distinctly rounded sclerotic knob, while the sclerotic knob in males was not often obvious. Uncertainty of the characteristics of the sclerotic knob makes versus the damaged of galeae it difficult to identify and describe species – especially when only a single-sex specimen is available. We recommend that future researchers: (1) collect multiple specimens to help ensure both adult males and females are collected (enabling further examination and study of the sclerotic knob across additional specimens and species); (2) carefully collect pseudoscorpions to avoid damaging the galeae; (3) cautiously examine the galeae particularly when specimens are few; and, (4) use scanning electron microscopy (e.g., Cokendolpher and Krejca 2010) to more accurately examine the structure of the galeae. The latter will enable researchers to determine whether galeae are reduced or altered during collection or transportation.

As with most Chinese hypogean pseudoscorpions (Table 1; Schawaller 1995; Mahnert 2003, 2009; Mahnert and Li 2016; Gao et al. 2017; Li et al. 2017; Gao et al.
Figure 18. Motian Cave, type locality of *Parobisium motianense* sp. nov. A Surrounding vegetation and agricultural areas with cave entrance (Red arrow)  B Entrance C Inside the cave entrance  D Stalactites E Cave landscape.

2018; Feng et al. 2019; Li et al. 2019), these four new species are currently considered single cave endemics. However, this may be due to limited investigations in the region, rather than actual short-range distributions of these species. There is a high density of caves in Guizhou – in particular, numerous caves occur within a 5 km radius of the caves containing these species. Specifically, there are at least three caves with a 5 km radius of Zhaorou Cave, no fewer than seven caves with a 5 km radius of the Sanlou Cave, and at least 10 caves with a 5 km radius in Biyun Cave. None of these caves have been inventoried for cave-dwelling arthropods. As a result, it is possible our newly described species are actually restricted to a geological formation rather than a single cave (Schawaller 1995; Mahnert 2009; Mahnert and Li 2016; Table 1). Additional investigations will be required to make this distinction.

While it is becoming increasingly well-established that caves in China are rich in cave biological resources and support subterranean-adapted species with highly restricted distributional ranges, the county presently lacks policies or a governmental agency to protect and manage subterranean natural resources. This presents challenges for conservation and management of sensitive subterranean animal populations. Unfortunately, human activities (i.e., urbanization, mining, and other related activities) in karst areas, and the development of tourist caves, continue without prior evaluations of the potentially sensitive natural resources and/or the biodiversity they may support.
Cave-dwelling pseudoscorpions of China

Figure 19. Zharou Cave, type locality of Parobisium qiangzhuang sp. nov. A entrance B Area where P. qiangzhuang specimens were collected.

(Whitten 2009). Therefore, we recommend the distributions of the new species described here be more thoroughly established through sampling of the abovementioned caves surrounding the type localities. Importantly, once we determine whether these species are single cave or regional endemics, this information may be used to guide management policies to protect these animals and their habitats.

Local human activities (Figs 20A, D, 21D) are more likely to have significant impacts on these cave-dwelling species, which could result in their imperilment or potentially their extinction. In fact, the four new Guizhou pseudoscorpions and their habitats are directly threatened by human activities. All four caves were close to human settlements and/or agricultural areas (within 0 m to 100 m) and were affected to varying degrees by other human activities. Motian Cave is surrounded by agricultural activities. For Zharou Cave, agricultural activities are less than 100 m away. In both cases, pesticide and fertilizer residues may contaminate the caves via runoff (Castaño-Sánchez et al. 2019). Although the entrance of Zharou Cave is somewhat obscured by vegetation, local residents are aware of this cave, and we observed recent evidence of human activities. The entrance of the Sanlou Cave is about 50 m from sand mining operations, and the deepest part of the cave has been modified and converted to a reservoir. Additionally, the sand mining facility and the water extraction activities may ultimately affect the survival of P. sanlouense sp. nov.

Biyun Cave, located in Biyun county park, is a tourist cave. Evidence of human activity was observed throughout the cave, which included refuse, remnants of bonfires, and graffiti on the cave walls. Subsequently, cave habitats have been damaged to varying degrees. During our work, we observed at least 10 tourists visiting the cave. Fortunately, because of the muddy and steep path at the back of the cave, we suspect fewer visitors will be willing to access the area where we found P. tiani sp. nov. As a result, this habitat may be somewhat protected.

Extinction is often characterized by time lags, and at-risk populations may persist for long periods of time near extinction thresholds prior to becoming extinct (e.g., Brooks et al. 1999, Hanski and Ovaskainen 2002, Vellend et al. 2006). These “ex-
tinction debts” (see Tilman et al. 1994) may occur when populations become isolated following significant environmental perturbations or intensive human activities. Both P. sanlouense sp. nov. and P. tiani sp. nov. occur in areas of intensive human activities. This further emphasizes the need for additional surveys to determine whether these species are single cave or regional endemics. This distinction will be of critical importance in determining the sensitivity of these animals to current human activities within and at proximity to the caves where they occur. Moreover, understanding their distributions will be required to develop effective monitoring protocols – if additional evidence supports that either (or both) species are single cave endemics.

There are other measures that should be examined to protect sensitive cave-dwelling species and their habitats. An outreach campaign to help educate villagers, school children, and tourists concerning the vulnerability of cave biological resources should be considered (refer to Mammola et al. 2019). At Biyun Cave, it may be worth posting educational signs (within the village and perhaps near the entrance) to explain the sensitivity of cave natural resources and that endemic species occur within, as well as guidelines for reducing human impacts to sensitive cave resources.
As research on cave biological resources in southern China continues, numerous additional new species with restricted ranges will be described. Understanding their distributions and the functional roles they play in these often highly sensitive ecological communities will be of paramount importance for developing management plans to protect both sensitive species and their habitat. Through these and other efforts, we hope our findings and data collected in the future will be employed to help shape effective cave resource management in China.

Acknowledgments

We are grateful to Mingyi Tian for contributing specimens and providing us with information and images (Figs 2, 18), on the caves he sampled. This work was supported by the National Natural Science Foundation of China (No. 31872198), and the Ministry of Science and Technology of the People’s Republic of China (MOST Grant No. 2015FY210300).
References


Cave-dwelling pseudoscorpions of China


First record of Pisidium subtruncatum Malm, 1855 (Bivalvia, Sphaeriidae) in an African cave

Hanane Rassam¹, Soumia Moutaouakil¹, Hassan Benaissa¹, Christian Albrecht², Mohamed Ghamizi¹

¹ Muséum d’Histoire Naturelle de Marrakech, Laboratoire Hydrobiologie, Ecotoxicologie, Assainissement et Changements globaux, Université Cadi Ayyad, Marrakech, Morocco ² Department of Animal Ecology & Systematics, Justus Liebig University Gießen, Heinrich-Buff-Ring 26 (IFZ), 35392, Gießen, Germany

Corresponding author: Hanane Rassam (hananerassam@gmail.com)

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Abstract
Studies on the bivalve family Sphaeriidae in North Africa are very limited at the surface water level, but even more for caves. During an expedition in 2019 to the Ait M’hamed cave (Oum Er Rabia Basin), six specimens of the genus Pisidium were collected. Morphometric and genetic analyses showed that these individuals belong to the species Pisidium subtruncatum Malm, 1855. This work is the first step towards future exploration of cave Sphaeriidae in North Africa.

Keywords
Molluscs, Subterranean, Invertebrates, Biospeleology, Ait M’hamed, Morocco

Introduction
Pisidium is a genus of freshwater bivalves belonging to the family Sphaeriidae that includes the smallest bivalves on Earth. Despite their small size, Pisidium species can be used for bioindication studies (Horsák 2001) and the usefulness of these species as markers of metal and organic pollution has been proved repeatedly (e.g. Ingram et
al. 1953; Wurtz 1955; Anderson 1977; Gadzała-Kopciuch et al. 2004; Alhejoj et al. 2017). The group is cosmopolitan and occurs in temporary and permanent aquatic environments. Along with Dreissenidae, Sphaeriidae is the only family of bivalves inhabiting subterranean habitats (Culver 2012; Prié 2019). Their occurrence in caves has been reported by a number—albeit few—of authors from different localities (e.g. *Pisidium hallae* Kuiper, 1983, *Sphaerium tasmanicum* Tenison Woods, 1876 from Australia (Kuiper 1983; Korniushin 2000), *Pisidium zoctanum* Poli, 1876 and *Pisidium crimeana* Stadnichenko, 1980 from Ukraine (Vargovitsh and Anistratenko 2016; Vinarski and Kantor 2016), *Pisidium casertanum* Poli, 1791 and *Pisidium personatum* Malm, 1855 from Scotland (Knight and Wood 2000; Knight 2018) and *Pisidium ljovushkini* Starobogatov, 1962, *P. cavatica* Zhadin, 1952 and *P. subterranea* Zhadin, 1932 from Caucasus (Vinarski and Kantor 2016)). In North Africa, studies on the freshwater clams of caves are lacking. In fact, in Morocco, even fewer studies are limited to the distribution of *Pisidium* species were seven species are reported (Kuiper 1972) and where extreme environments such as caves are not prospected. The aim of this paper is to report for the first time the occurrence of a Sphaeriidae species in a Moroccan cave.

**Material and methods**

In May 2019, we prospected the Ait M’hamed cave. This cave is located in Oum Er Rabia basin at 1693 m of altitude (31°52’48”N, 06°27’02”W). The cave is dug at the bottom of a cliff in the calcareous of Bajocian – Bathonian period with horizontal stratification (Doat et al. 2005). The water flowing inside the cave is drained from a spring since it is permanent water even during dry season and expeditors reported the continuity of flowing tributaries even after more than 1500 m from the cave entrance (Doat et al. 2005). The entrance to the cave is wide, about 5 m large and 2.50 m high (Fig. 1A). Physical-chemical parameters of the water were measured at two points, the cave entrance and the waterfall (Table 1) using a multiparameter tool (HI98194 portable probe).

The sampling was carried out with a sieve of 200 μm of diameter in muddy sediments and lead to the collecting of 6 specimens belonging to the genus *Pisidium* (Fig. 1B, C). The maximum distance explored of the cave is 4000 m, however, only 3052 m were topographically mapped (Fig. 2). The specimens were collected at two points: one at 100 m and the second at 500 m from the entrance. Specimens collected were placed in 80% ethanol for morphological and genetic analysis. No permit for sampling was required.

In the laboratory, the identification of the specimens was based on morphological characters following the descriptions of Adam (1960) and Killeen et al. (2004) using a stereomicroscope (Leica Microsystems CH 9435 Loupe). On the basis of the scaled images of the shells obtained with the stereomicroscope, we used TpsDig v. 2.31 (Rohlf 2005) to produce the following shell measurements for a better morphological diagnosis: L (shell length), H (shell height), LP and LA (length of posterior and anterior parts respectively), LL (length of ligament), LE (umbo length), LH (hinge length)
First record of Pisidium subtruncatum Malm, 1855 in an African cave

Figure 1. Study area Ait M’hamed cave a The cave entrance b the sampling and c the inside of the cave (Moutaouakil 2019).

Table 1. Measurements of physical and chemical parameters at two localities in the cave system (see Fig. 2, May 2019).

<table>
<thead>
<tr>
<th></th>
<th>H (%)</th>
<th>T(°C)</th>
<th>T(°C) of water</th>
<th>Dissolved oxygen. (mg/l)</th>
<th>Conductivity (µS/com)</th>
<th>pH</th>
<th>Nitrites (g/mol)</th>
<th>Phosphate ion (g/mol)</th>
<th>Ammonium (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cave entrance</td>
<td>28</td>
<td>20.7</td>
<td>20</td>
<td>5.32</td>
<td>421</td>
<td>7.2</td>
<td>0.08</td>
<td>0.11</td>
<td>0.03</td>
</tr>
<tr>
<td>Waterfall</td>
<td>28</td>
<td>19.1</td>
<td>21.6</td>
<td>4.65</td>
<td>432</td>
<td>7.09</td>
<td>0.071</td>
<td>0.06</td>
<td>0.05</td>
</tr>
</tbody>
</table>

and HH (hinge height). The mean shape of the shells was obtained on the basis of semi landmark coordinates plotted with TpsRelw v. 1.70 (Rohlf 2003) (Fig. 4).

Soft bodies were extracted for genetic analysis in order to confirm morphological identification. DNA isolation followed a CTAB protocol (Wilke et al. 2006). Amplification of mitochondrial gene fragments which are regularly used in sphaeriid barcoding and phylogenetics was unsuccessful. Therefore, Polymerase Chain Reaction after 9 cycles running for 1.5 h was performed with thermocycler Eppendorf Mastercycler using the nuclear gene H3 and primers of Colgan et al. (2000). Sequencing was carried out on an ABI 3730 at LGC Genomics, Berlin, Germany. Resulting sequences were checked in the NCBI database using nucleotide BLAST (BLASTn suite: megablast) returning highly similar sequences stored in the NCBI GenBank database (Zhang et al. 2000). The top five BLAST hits (sorted by max score; default) for each individual are shown in Table 3.
Figure 2. Cave topography. Red points: Sampling localities (green crosses included), green crosses: *P. subtruncatum* occurrence.

Figure 3. Two specimens of *P. subtruncatum* from Ait M’hamed cave *a, d* external view of the shell of the left and right sides of both specimens *b, e* dorsal view of both specimens *c, f* internal view of left and right valves of both specimens.
First record of Pisidium subtruncatum Malm, 1855 in an African cave

Results and discussion

Morphometric results of the four specimens collected showed that they have a length ranging between 3.49 and 1.91 mm and height between 2.93 and 1.62 mm. The shell is silky with slight striations and the umbo is narrow and located posteriorly. The shape of the shell is sub-angulated, the most extreme point of the anterior part is located lower than the middle of the shell height (Figs 3, 4). The anterior part is clearly longer than the posterior part (see measurements on Table 2). The hinge is thicker, more or less wide. The ligament pit is long. The left valve with two long cardinal teeth, the lower (C₂) and the uppermost (C₄) parallelly located, C₄ overlaps C₂ at anterior end, C₃ is long and slightly curved (Fig. 5). All individuals found in the present work are exactly similar

Table 2. Measurements of internal shell features.

<table>
<thead>
<tr>
<th>N</th>
<th>L ± SD</th>
<th>H ± SD</th>
<th>LA ± SD</th>
<th>LP ± SD</th>
<th>LE ± SD</th>
<th>LL ± SD</th>
<th>LH ± SD</th>
<th>HH ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>4</td>
<td>2.96 ± 0.81</td>
<td>2.28 ± 0.53</td>
<td>1.8 ± 0.64</td>
<td>1.16 ± 0.31</td>
<td>0.81 ± 0.24</td>
<td>0.48 ± 0.09</td>
<td>1.44 ± 0.38</td>
</tr>
</tbody>
</table>

Table 3. List of the first five significant BLAST hits (NCBI GenBank accessed on 15/06/2019).

<table>
<thead>
<tr>
<th>Description</th>
<th>Max score</th>
<th>E value</th>
<th>Percent identity</th>
<th>Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pisidium subtruncatum isolate 17469 histone 3 (H3) gene, partial cds</td>
<td>599</td>
<td>3e-167</td>
<td>99.39%</td>
<td>KU376244.1</td>
</tr>
<tr>
<td>Pisidium atkinsonianum isolate 6024 histone 3 (H3) gene, partial cds</td>
<td>595</td>
<td>3e-166</td>
<td>99.39%</td>
<td>KU376227.1</td>
</tr>
<tr>
<td>Pisidium viridarium isolate 15834 histone 3 (H3) gene, partial cds</td>
<td>590</td>
<td>2e-164</td>
<td>99.09%</td>
<td>KU376246.1</td>
</tr>
<tr>
<td>Pisidium personatum isolate 17456 histone 3 (H3) gene, partial cds</td>
<td>590</td>
<td>2e-164</td>
<td>98.78%</td>
<td>KU376241.1</td>
</tr>
<tr>
<td>Pisidium casertanum isolate 17462 histone 3 (H3) gene, partial cds</td>
<td>586</td>
<td>2e-163</td>
<td>98.78%</td>
<td>KU376228.1</td>
</tr>
</tbody>
</table>

Figure 4. Mean overall shell outline shape of the four adult specimens of P. subtruncatum. The mean shape was generated from semilandmarks coordinates of the right valves using the tpsRelw.

Results and discussion

Morphometric results of the four specimens collected showed that they have a length ranging between 3.49 and 1.91 mm and height between 2.93 and 1.62 mm. The shell is silky with slight striations and the umbo is narrow and located posteriorly. The shape of the shell is sub-angulated, the most extreme point of the anterior part is located lower than the middle of the shell height (Figs 3, 4). The anterior part is clearly longer than the posterior part (see measurements on Table 2). The hinge is thicker, more or less wide. The ligament pit is long. The left valve with two long cardinal teeth, the lower (C₂) and the uppermost (C₄) parallelly located, C₄ overlaps C₂ at anterior end, C₃ is long and slightly curved (Fig. 5). All individuals found in the present work are exactly similar
to the description given by other authors (for a review see Adam 1960; Piechocki 1989; Killeen et al. 2004). Moreover, the identification was also confirmed by a specialist researcher who is familiar with Pisidium (M. Zettler Warnemünde 2019, in litt.).

Figure 5. Position and shapes of cardinal teeth and ligament pits in left (a) and right valve (b). c: cardinal teeth.
Genetic results did not contradict the identification of the species as *P. subtruncatum* and, as presented in the list of significant BLAST hits (Table 3), the five first sequences with the highest similarity with our sequences are *Pisidium subtruncatum*, *Pisidium atkinsonianum* Theobald, 1876, *Pisidium viridarium* Kuiper, 1956, *Pisidium personatum* and *Pisidium casertanum*, all from Nepal (Boessneck et al. 2016). With all uncertainty related to the conservative nature of the marker H3, these results (max score 599, see Table 3) support the morphological determination of the cave specimens as *P. subtruncatum*.

*P. subtruncatum* was already recorded in a river of the Sebou basin (Kuiper 1972) (Fig. 6), but no published studies cited the presence of this species in the Oum Er Rbia basin. The IUCN conservation status of this species in North Africa is considered as endangered because of its restricted area of occupancy and declining quality of habitat (García et al. 2010). The four individuals collected were from two localities and they inhabited a dark and muddy environment with no sign of anthropogenic influence. The water depth did not exceed 1 m and its overall quality is assessed as good (Table 1) (ONSSA 2018). The ecology of the genus *Pisidium* is resulting in surprising flexibility
as outlined by the current finding of a species living in the solid interstitial environment in Germany (Groh et al. 2020). *Pisidium subtruncatum* is an euryecious species with a palearctic distribution, inhabiting different kinds of habitats, its optimum conditions are met in small rivers with sandy-muddy substratum (Piechocki 1989), especially when being concentrated with macro-ions and organic matter (Besperalaya 2015). This agrees with our findings (e.g. high conductivity). The influence of darkness was not considered for the present note; however, it is known that all bivalves have light-sensitive cells (Cofransesco 2002) and the impact of light on bivalves growth had been proved by Medcof and Kerswill (1965).

**Conclusion**

In general, the Sphaeriidae family is neglected in North Africa and studies on this group of benthic organisms are very limited compared to other taxa. The originality of this work consists in the recording for the first time of a member of the Sphaeriidae family in an African cave and to our knowledge the first record of *P. subtruncatum* in a cave. Studies such as ours reported here should be expanded to other caves in Morocco (Fig. 6). This is important in order to enhance our faunal knowledge and to determine the actual conservation status of *Pisidium* species. Moreover, this need becomes urgent given the increasing human pressure including habitat loss and anthropogenic transformation of habitats of *Pisidium* species (e.g. rivers, lakes and springs) in a Mediterranean biodiversity hotspot region such as Morocco.

**Acknowledgment**

We thank Dr. Michael Zettler (Warnemünde, Germany) for confirming the identification of the species.

**References**


Acheroxenylla (Collembola, Hypogastruridae), first record from the Americas with description of a new species from a Peruvian cave

José G. Palacios-Vargas

Laboratorio de Ecología y Sistemática de Microartrópodos, Depto. Ecología y Recursos Naturales, Facultad de Ciencias, UNAM, Coyoacán 04510, México

Corresponding author: José G. Palacios-Vargas (troglolaphysa@hotmail.com)

Abstract
A new diagnosis for Acheroxenylla Ellis, 1976 is proposed, based on new characteristics recently discovered in other species of the genus. A new species living on guano from oil bird guacharo is described and illustrated and its Barcode Index Number (BIN) from BOLD System is given. A key for the identification of the four known species is also included.

Keywords
Xenyllian group, cueva de Samuel, chaetotaxy, troglomorphy

Introduction
Ellis (1976) created the genus Acheroxenylla as closely related to Xenylla. Since they share the absence of postantennal organ, the number and location of Ant. IV sensilla, the number of tenent hairs on tibiotarsi, the absence of unguiculus, and the general...
appearance of the chaetotaxy. He argued that most *Xenylla* species have 5 + 5 eyes or at least 4 + 4. So, he created a new genus for one species from Crete, Greece, which most remarkable characteristic is the presence of only two eyes per side and a complete lack of furcula. However, even in *Xenylla* the reduction or absence of a furcula is rare, there is a gradation between fully developed furcula (with manubrium, dens and mucro) and completely lacking, as seen from *X. boerneri* Axelson, 1905 to *X. acauda* Gisin, 1947. Later Fjellberg (1992) found two species of *Acheroxenylla* in Canary Islands, one of them, *A. furcata* with a reduced furcula and, thus, the author increased the knowledge and characters of the genus.

After Thibaud et al. (2004), *Acheroxenylla* has the type I of chaetotaxy in the Hypogastruridae with short setae (mesosetae), sometimes more or less ciliated, and with longer and fine sensory setae; and these authors have called it type “Xenyllian” because all of them lack a postantennal organ. There are 8 genera in this type: *Acherongia*, *Acherontides*, *Acherontiella*, *Acheroxenylla*, *Paraxenylla*, *Pseudacherontides*, *Thibaudylla* and *Xenylla*, with almost 200 species of the 715 known in the family Hypogastruridae (Bellinger et al. 2019). Around 130 Collembola species in 12 families have been cited from Perú (Bocanegra 2013), two of them members of the Xenyllian group but Peruvian members of this group living in caves have never been studied.

**Material and methods**

In a recent expedition organized by Josiane Lips to collect cave fauna, several samples were collected in six caves from Rioja Province (northern Perú). They were processed by Berlese-Tullgren, hand collected and kept in alcohol. All of them were preserved in ethanol 96% at the Department of Entomology, Museo de Historia Natural, Universidad Mayor de San Marcos, Perú. Later the specimens of Collembola were sent to the Laboratorio de Ecología y Sistemática de Microartrópodos, Faculty of Sciences, Universidad Nacional Autónoma de México for study.

Among the Collembola, some specimens of *Acheroxenylla* were found. Five were preserved for molecular study and six were prepared in Hoyer’s solution. Later, they were studied under a contrast phase microscope Carl Zeiss mod. 465270-9906 and drawn with the aid of a camera lucida.

For the molecular study each of the five specimens were photographed and sent for sequencing with the standard COI–5P marker (“DNA barcode”, Ratnasingham & Hebert, 2013) at the Canadian Centre for DNA Barcoding.

Abbreviations used in this paper are: Ant = antennal segment; Abd = abdominal segment; hr = anal valve setae; PAO = postantennal organ; Sgd = dorsal guard sensillum; Sgv = ventral guard sensillum; Th = thoracic segment; a = anterior row of setae; m = median row of setae; m’ = microsensillum; or = apical organ; p = posterior row of setae; S = sensillum; ss = sensorial seta; Tita = tibiotarsus.
Results

Class Collembola Lubbock, 1870
Order Poduromorpha Börner, 1913
Family Hypogastruridae Börner, 1906

Acheroxenylla Ellis, 1976 new modified diagnosis

Notes. Small Hypogastruridae (from 0.5 to 1.3 mm). Setae not differentiated in mac- rosetae, only with smooth or slightly barbulate mesosetae and longer sensory setae. They are usually white, with dark or black color only under each eye, with possible bluish gray pigmentation all over the body. Antennae cylindrical about the same length as the head, with a simple or trilobed retractile papilla; Ant IV with a dorso-external microsensillum, a subapical sensorial organ and 4 cylindrical or oval sensilla: 3 external and one internal. Ant. III organ with 2 microsensilla hidden by or not by a tegumentary fold and framed by 2 longer guard sensilla. Ocelli 2 + 2 (sometimes 1 + 1). Postantenal organ always lacking. Head chaetotaxy with setae a0 and d0; five pairs of dorsal cephalic setae; and two or three subdorsal pairs. Claws without teeth and empodium. Th I with 3 + 3 setae; Th II–III with 4 + 4 setae on row a, 3 + 3 on m row and 4 + 4 on row p. Or a: 3 + 3 and 2 + 2; m: 3 + 3 (m2 and m3 absent) and 1 + 1 lateral; p: 4 + 4 or 5 + 5; two pairs of ss at position m6 and p4. Without lateral microsensillum on Th II. Abd I–III with 2 rows of setae (a and p): 5 + 5 or 6 + 6; one pair of ss at p5; Abd IV with 3 rows of setae (a: 3 + 3 or 4 + 4; m: 2 + 2 (with or without m1 or m4); p: 4 + 4 or 5 + 5; one pair of ss at position p5). Abd V with 2 rows (a and p: 2 + 2 or 3 + 3) one pair of ss at position p3. Each tibiotarsus with 2 or 1 tenent hairs. Ventral tube with 4 + 4 setae. Retinaculum either with 2 or 3 teeth on each ramus or complete absent. Furcula present, reduced or absent. Manubrium, when present, with 2 or 3 pairs of setae, dens if present short or long with maximum 2 setae. Mucro absent except one case. Always with 2 very small anal spines on papillae of the same size.

Type species. Acheroxenylla cretensis Ellis, 1976

Key to species

1  Furcula and retinaculum absent ......................................................... 2
   – Furcula and retinaculum well developed or reduced, but always present ..... 3
2  2 + 2 eyes, occasionally with small pigment spots; p3 on Abd. IV present.....
   .................................................................................A. cretensis Ellis, 1976 (Greece: Creta Island)
   – 2 + 2 eyes, always with blue pigment spots; p3 on Abd. IV absent
   .................................................................................A. canariensis Fjellberg, 1992 (Spain: Canary Islands: El Hierro, Gomera, La Palma, Tenerife, Gran Canaria, Lanzarote)
Retinaculum with 3 + 3 teeth. Furcula well developed with mucro, long dens with 2 long setae... *A. lipsae* sp. nov. (Perú: Province Rioja, Cueva de Samuel)

Retinaculum with 2 + 2 teeth. Furcula very reduced without mucro, short dens with 2 tiny setae............. *A. furcata* Fjellberg, 1992 (Spain: Canary Islands: La Gomera, Tenerife, Gran Canaria, Fuerteventura, Lanzarote)

*Acheroxenylla lipsae* sp. nov.
http://zoobank.org/3EF8532D-FD4F-4DB2-A547-5C60CE1107C5
Figures 1–12

**Description.** *Holotype female* (number FC-UNAM 22501) and one paratype female (numbers FC– UNAM 22502) are kept at Dept. Entomology; Museo de Historia Natural, Universidad Mayor de San Marcos, Perú; two paratypes females one male and one juvenile (numbers FC–UNAM 22500, 22503 to 22325) are kept at Mexican Collembola collection at Facultad de Ciencias, UNAM.

**Type locality.** Perú: Region San Martín; Province Rioja, Cueva de Samuel (6°06'92"S, 77°31'58"W) 1,720 m a.s.l. About 5.5 Km North-West of town Naciente de Rio Negro. 16-viii-2017, sample 14470, J. Lips col.

**Diagnosis.** *Acheroxenylla lipsae* sp. nov. is characterized by the presence of a well-developed furcula with mucro, long dente with two long dental setae each, three manubrial setae and a retinaculum with three teeth. Tibiotarsi are longer than in other species known.

**Description.** **Body length** (average of 7 specimens) = 1.25 mm. Setae not differentiated in macro and microsetae, all smooth and sharp mesosetae about 11 μm with small barbulations. Sensorial setae longer than regular setae, about 30 μm. Sensorial formula as 022/11111. Color, some specimens (Figures 1, 2) with very dark eyespots; others gray with small patches of blue color on body and black eyespots (Figures 3, 4). Cuticular granulation strong, Yossi’s parameter 5 or 6. Ratio of head: antenna = 1:0.8 labrum formula: 2/5,5,4.

**Ant I** with seven dorsal setae, Ant II with 12 setae. Ant III with 17 setae in two whorls, sense organ with two free club-shaped microsensilla, not covered by tegumentary fold; two short guard sensilla (Sgd and Sgv) of same shape and size, and one ventral microsensillum. No eversible sac between Ant III-IV. Ant IV with four cylindrical sensilla, one dorsal and three latero-external; subapical organite, lateral microsensillum and simple subapical bulb (Figure 5), no sensory file on ventral side. Ratio of Ant I: II; III–IV = 1:1.1; 1.4; 3.8.

**Head** with typical chaetotaxy for the genus, similar to *Xenylla*, only 3 subdorsal setae, seta c1 and only 1 setae v (v1), and 3 subequal setae in ocular area (Figure 6). 2 + 2 eyes of about equal diameter with very strong granulations of dark pigment. PAO absent. Labium with 4 + 4 setae (one longer than others); 3 pairs of postlabial setae. Mandible with 3–4 apical teeth, and normal molar plate. Maxilla with six lamellae. Th I with 3 + 3 dorsal setae and 1 + 1 lateral on upper subcoxae. Each Th II and III with 3 irregular rows of setae (Figure 8), sensorial setae m6 and p4 as usual.
Leg chaetotaxy from I to III: precoxae 0,1,2; coxae 3,6–8,5–8; trochanters 5,5,4; femora 12,11,10 one ventral seta very long, as acuminate tenent hair; Tita 19,19,18 (Figures 7, 9); pretarsi 2,2,2. Two dorsal tenent hairs weakly clavate on dorso-distal whorl on Tita I and II; one on Tita III. Unguis thin, elongated, curving slightly, without any tooth (Figures 7–9). No unguiculus. Ratio Tita/unguis = 1:1.

Dorsal chaetotaxy of abdomen as in Figure 10. Abd I–III with 2 irregular rows of dorsal setae, 1 sensorial seta on P5, except Abd V with p3 as sensorial seta. Number of axial setae from Abd I to III is 2 + 2: Abd. IV with 3 + 3; Abd. V with a1 and a2, p3 is ss. Abd VI with 2 rows of setae, a1-3, p1 modified as spine, p 2-3 normal setae. Two small anal spines, as short as their tubercle.

Ventral chaetotaxy. Thoracic sternites and Abd I without setae. Ventral tube with 4 + 4 setae. Abd II with 9 – 12 setae, one of them (p3) very long; Abd III with 6 setae, p3 slightly longer than others. Abd IV dorsolateral with 5 setae, one of them very long (Figure 10). Retinaculum with 3 + 3 teeth, without seta on corpus (Figure 10). Furcula well developed. Manubrium with 3 pairs of setae of same length. Dens dorsally with moderate granulation and with 2 subequal setae, with a smooth elongated area on anterior part of dens. Mucro more than the half-length of dens, long and narrow with one small outer lamella, apex curved and more sclerotized (Figure 11). (Ratio Manubrium: dens; mucro = 1:0.8; 0.5). Mucro better delimited on anterior part by a clear notch (Figure 12). Genital plate of female with 3+3 pregenital, 10–13 circumgenital

Figures 1–4. 1 Acheroxenylla lipsae sp. nov. 1, group of specimens floating on water close to the guano of oil birds 2 two specimens dorsal view just collected in ethanol 96%; photos 1 and 2 by Josiane Lips 3 dorsal view 4 lateral view, photos 3 and 4 by Maira Montejo and Angela Arango, scale in photos 3 and 4 is 150 μm.
Figures 5–7. *Acheroxenylla lipsae* sp. nov. 5 ant IV dorsal view 6 head chaetotaxy 7 Tita I.

and 1 + 1 eugenital setae. Genital plate of male with 3 + 3 pregenital, 44 circumgenital and 4 + 4 eugenital setae. Each anal valve with 13 regular and 1 hr setae.

**Variation.** Some asymmetries on body chaetotaxy were observed. Several specimens have setae somehow displaced, giving the appearance of asymmetries. One case
Acheroxenylla (Collembola, Hypogastruridae) from a Peruvian Cave

Figures 8, 9. Acheroxenylla lipsae sp. nov. 8 dorsal chaetotaxy of Th. I – III 9 Tita III.

of supernumerary setae on left side of abdominal segment III was observed on one paratype, where there were 3 setae “p4”, giving the appearance of sensorial seta to be on position “p7”.

Etymology. This species is dedicated to Josiane Lips, for her contribution to the knowledge of cave fauna from Perú, Mexico and many other places.

Discussion. This new species is the largest and most pigmented member of this genus. The main differences of Acheroxenylla lipsae sp. nov. and the other species
is the presence of a well-developed furcula with mucro, long dens and two long dental setae, 3 + 3 manubrial setae and retinaculum with 3 + 3 teeth. *A. furcata* has a reduced furcula, with no manubrial setae, very short dens with one seta each and mucro absent; its retinaculum has only two teeth on each ramus. The type species *A. cretensis* and *A. canariensis* lack completely the furcula and retinaculum. All the species have only two eyes per side; nevertheless, in the new species *A. lipsae* sp. nov., eyes are better developed and closer to each other, and that is why their position seems to be “D” and “E”. After the drawings of Ellis (1976), the eyes of
A. cretensis are "B" and "E"; as pointed by Ellis (1976): “only 2 + 2 eyes small, widely separated ocelli”. Another difference is that A. cretensis has only 2 subdorsal cephalic setae, while the new species has 3 pairs, setae sd5, sd4 and sd3, similar to A. furcata. There are small differences in the head chaetotaxy, A. lipsae sp. nov. has cephalic setae a0, as A. cretensis.

Figures 11, 12. Acheroxenylla lipsae sp. nov. 11. Ventral abdominal chaetotaxy from Abd. III to V, with retinaculum and furcula; 12. two mucrodens in lateral position.
About the pigment, *A. furcata* is white with small spots under each of the 2 + 2 or 1 + 1 eyes and sometimes a scattered bluish gray pigment is present all over the body, while *A. lipsae* sp. nov. is more pigmented. Tibiotarsi of *A. furcata* with one apical tentent hair (A1), sometimes weakly clavate, is similar to the new species. The ungu of the Peruvian species is more elongated than in any other species of the genus (ratio tibiotarsus: ungues: 1: 1.0), so this may be a troglomorphic character, and also in the other species tibiotarsus is about twice the length of ungu.

**Molecular results.** DNA was successfully obtained from three specimens, sequences BCICL008–19 (length 620bp), BCICL009-19 (657bp) and BCICL010 (632bp), which were deposited in the project BCICL of the Barcode of Life Data System (http://www.barcodinglife.org/index.php). Cuticles of two specimens were recovered and mounted in Hoyer's solution which represent the vouchers and are kept at the author's institution as type material.

**Description of the cave.** The type locality is Cueva de Samuel. Collecting was done 200 m from the entrance on Guácharo guano, where was the new species found. It is an active cave with water flowing from the cave. The air temperature was 15 °C. Its entrance is about 1,720 m a.s.l. In the first part of the cave there is a gallery with a colony of oil birds (*Guácharos, Steatornis caripensis* Humboldt, 1817). 500 m deep in the cave there is a big room with many big stalagmites, named Chachapoyas room. There are two small waterfalls, a small at 800 m from entrance and more far another of 10 m. One of the tunnels finishes in a well of more than 40 m.

**Other Collembola in the area.** There were 20 springtails found in the region of Rioja. In two caves only one species was found in each: Cueva del Lobo Perdido (*Pseudosinella* sp.) and Tragadero de Bellavista (*Cyphoderus* sp.). Two other caves had three Collembola: Piedra Brillante (*Trogolaphysa* sp., *Pseudosinella* sp., *Pararrhopalites*) and Cueva de los Loros (*Pseudosinella* sp., *Dicranocentrus* sp., *Trogolaphysa* sp.). In Cueva Palestina there were four species: (*Pseudosinella* sp., *Folsomia* sp., *Folsomiella caeca* (Folsom, 1927), *Folsomides troglobius* (Rapoport & Maño, 1969) while Cueva de Samuel was the most diverse with a new species of *Acheroxyella*, and also specimens of *Folsomina* sp., *Pseudosinella* sp., *Trogolaphysa* sp., *Cyphoderus* sp., *Isotomurus* sp., *Pararrhopalites ecuadorensis* Bretfeld et Trinklein, 2000.

*Folsomiella caeca* (Folsom, 1927) was described from limestone caves in bat dung at Panama. Later it was found at Ecuador in guano from several caves by Najt and Thibaud (1987). As it also was collected by one of them in Venezuela and Peru, they have considered this species to edaphic - troglobile. *Folsomides troglobius* (Rapoport et Maño, 1969) was originally described from one cave close to Araira, and Cueva del Guácharo in the state of Miranda, Venezuela. Later was cited by Najt and Thibaud (1987) from the cave of Barberanes in Ecuador. This is the first time that both species has been cited from Cueva Palestina in Perú.

In Cueva Samuel, besides the new species, there were other six Collembola found in different samples. Among them, *Pararrhopalites ecuadorensis* Bretfeld et Trinklein, 2000 was described from one cave from Otonga (Cotopaxi), Ecuador and now is found for the first time from Peru.
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