RESEARCH ARTICLE



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

Olivia F. Boyd^{1,2}, 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)

Academic editor: O. T. Mold	wan Received 5 September 2019 Accepted 10 February 2020 Published 10 March 2020

Citation: Boyd OF, Philips TK, Johnson JR, Nixon JJ (2020) Geographically structured genetic diversity in the cave beetle *Darlingtonea kentuckensis* Valentine, 1952 (Coleoptera, Carabidae, Trechini, Trechina). Subterranean Biology 34: 1–23. https://doi.org/10.3897/subtbiol.34.46348

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 Darlingtonea 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_{ST}) 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_{CT}), low variability among populations within clusters (Fsc), 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 Darlingtonea 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.

Copyright Olivia F. Boyd et al. This is an open access article distributed under the terms of the Creative Commons Attribution License (CC BY 4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

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.



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).

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.

Study species

Darlingtonea 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 cavespecialized carabids of the subtribe Trechina, Darlingtonea are true troglobionts, with adaptations for subterranean life: they lack eyes and wings, possess enlarged mouthparts, lengthened appendages, and specialized sensory setae, and are depigmented compared with their epigean relatives (Fig. 2). Darlingtonea 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 *Darlingtonea kentuckensis*. *Darlingtonea 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



Figure 2. Gravid female *Darlingtonea kentuckensis* photographed in Fletcher Spring Cave, Rockcastle County, Kentucky. Photo courtesy of Dr. Matthew Niemiller, University of Alabama, Huntsville.

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 Saltpeter 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 troglobitic 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.



Figure 3. Cave localities of currently known sites for *Darlingtonea kentuckensis*. White dots were the caves sampled for this study while black dots represent caves unsampled.

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 *Darlingtonea* 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 *Geomyces 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 *Darlingtonea* 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.

Taxon	Cave	County	Collection	N	Faunal	Local Watershed/Code	GenBank accession
Code			Date		Region	(River Drainage)	number
BLO	Blowing	Wayne	1-Mar-2014	3	4	Otter Creek/OT (CR)	MN880837, MN880838, MN880839
CLF	Clifford Pearson	Estill	14-Aug-2014	2	1	Station Camp Creek/ SC (KR)	MN880814, MN880815
CLI	Climax	Rockcastle	31-Jul-2014	4	2	Roundstone Creek/RO (RR)	MN880810, MN880811, MN880812 MN880813
FLE	Fletcher	Rockcastle	15-Mar-2014	3	2	Skegg Creek/SK (RR)	MN880827, MN880828,
	Spring	D 1 1			-	n 1 0 1/00	MN880829
GSP	Great Saltpeter	Rockcastle	15-Aug-2014	4	2	Roundstone Creek/RO (RR)	MN880817, MN880818, MN880819, MN880820
HIC	Hicksey	Jackson	14-Aug-2014	4	1	Station Camp Creek/ SC (KR)	MN880806, MN880807, MN880808, MN880809
HIS	Hisel	Jackson	1-Aug-2014	1	1	Station Camp Creek/ SC (KR)	MN880805
HRT	Hurt	Wayne	12-Jul-2014	4	4	Beaver Creek/BE (CR)	MN880846, MN880847, MN880848, MN880849
JES	Jesse	Wayne	28-Sep-2013	4	4	Otter Creek/OT (CR)	MN880836, MN880840, MN880844, MN880845
JGR	John Griffin	Jackson	31-Jul-2014	4	2	Horse Lick Creek/HL	MN880801, MN880802, MN880803 MN880804
KOG	Koger	Wayne	28 Sep 2013	1	4	(RR) Beaver Creek/BE (CR)	MN880850
LAI	Lainhart #1	Jackson	1-Aug-2014	4	1	Station Camp Creek/	MN880798, MN880799,
		,	8			SC (KR)	MN880800, MN880816
LAK	Lakes	Jackson	31-Jul-2014	3	2	Horse Lick Creek/HL (RR)	MN880792, MN880796, MN880797
MOR	Morning Hole	Jackson	14-Aug-2014	2	1	Station Camp Creek/ SC (KR)	MN880794, MN880795
MUL	Mullins Spring	Rockcastle	15-Mar-2014	2	2	Roundstone Creek/RO (RR)	MN880821, MN880822
PHC	Pine Hill	Rockcastle	15-Mar-2014	3	2	Roundstone Creek/RO (RR)	MN880830, MN880831
PIN	Piney Grove	Pulaski	20-Oct-2013	3	3	Pitman Creek/PI (CR)	MN880855, MN880856, MN880857
POU	Pourover	Pulaski	20-Oct-2013	4	3	Buck Creek/BU (CR)	MN880858, MN880859, MN880860, MN880861
RCH	Richardson's	Pulaski	20-Oct-2013	4	3	Pitman Creek/PI (CR)	MN880866, MN880867, MN880868, MN880869
ROA	Roadside	Pulaski	4-Jul-2012	1	3	Pitman Creek/PI (CR)	MN880862
SAV	Savage (Copperas Saltpeter)	Clinton	28-Sep-2013	2	4	Spring Creek/SP (CR)	MN880834, MN880835
SOR	Sinks of Roundstone	Rockcastle	15-Aug-2014	2	2	Roundstone Creek/RO (RR)	MN880832, MN880833
SRI	Sinks and Rises	Jackson	1-Aug-2014	3	2	Horse Lick Creek/HL (RR)	MN880790, MN880791, MN880793
STA	Stab	Pulaski	4-Jul-2012	4	3	Buck Creek/BU (CR)	MN880851, MN880852, MN880853, MN880854
STL	Steele Hollow	McCreary	12-Jul-2014	3	4	Little South Fork/LS (CR)	MN880841, MN880842, MN880843
TEA	Teamers	Rockcastle	15-Aug-2014	4	2	Roundstone Creek/RO (RR)	MN880823, MN880824, MN880825, MN880826
WIND	Wind	Pulaski	4-Jul-2012	4	4	Pitman Creek/PI (CR)	MN880863, MN880864, MN880865, MN880870



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 withinpopulation 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 predefined 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.

 $\rm F_{ST}$ estimates the degree of differentiation among subpopulations within the total population. The closer $\rm F_{ST}$ is to 1, the greater the extent of allelic fixation or identity within populations (Holsinger and Weir 2009). $\rm F_{SC}$ estimates the differentiation among populations within the groups to which they are assigned. The closer $\rm F_{SC}$ is to 1, the more heterogeneity within groups. $\rm F_{CT}$ estimates differentiation among those groups of populations. The closer $\rm F_{CT}$ 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), $\rm F_{CT}$ should be high relative to $\rm F_{SC}$.

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 *a posteriori*, in contrast to the *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

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation	
AMOVA I					
Among groups	3	149.425	2.19461 (Va)	56.30	
Among populations within groups	23	108.884	1.44888 (Vb)	37.17	
Within populations	58	14.750	0.25431 (Vc)	6.52	
Total	84	273.059	3.89780	100	
Fixation Indices: I					
FSC	0.85069	Vb and FSC :	P(random > observe	ed) = 0.00000***	
FST	0.93476	Vc and FST : P(random < observed) = 0.00000*		ed) = 0.00000***	
FCT	0.56304	Va and FCT :	P(random > observe	ed) = 0.00000***	
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) = 0.0000		ed) = 0.00000***	
FCT	0.60846	Va and FCT :	P(random > observe	ed) = 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 > observe	ed) = 0.00000***	
FST	0.93644	Vc and FST : P(random < observed) = 0.00000***			
FCT	0.81935	Va and FCT :	P(random > observe	ed) = 0.00000***	

Table 2. AMOVA statistics, fixation indices, and results of hypothesis tests for structure hypotheses I (four faunal regions), II (ten watersheds), and III (five genetic clusters).



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. Overlain 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



Figure 7. Representative male genitalia from 17 of the sampled caves: I Wells Cave; 2 Pine Hill Cave;
3–5 Wind Cave; 6 Richardson's Cave; 7, 8 Lainhart #1 Cave; 9, 10 and 15, 16 Pourover Cave; 11, 12 John Griffin Cave; 13 Climax Cave; 14 Hicksey Cave; 17, 18 Stab Cave; 19, 20 Piney Grove Cave; 21, 22 Dykes Bridge Cave; 23 Great Saltpeter Cave; 24 Teamers Cave; 25 Mullins Spring Cave; 26 Jesse Cave; 27 Steel Hollow Cave. Note that Wells and Dykes Bridge Caves were not included in the genetic study.

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 lex-ingtoni* 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_{cT} 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_{cr} (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.

Hypothesis (group #)	Populations included	% variation explained by	$P_{obs>sim}(\alpha=0.05)$
0 1		geographic distance	
III (1)	CLF, HIC, LAI, MOR, HIS, SRI, JGR, LAK, CLI	<1	0.468
III (2)	FLE, PHC, SOR	<1	0.6637
III (5)	TEA, MUL, GSP	<1	0.673
I (1)	CLF, HIC, LAI, MOR, HIS	7	0.761
I (2)	SRI, JGR, LAK, CLI, MUL, GSP, TEA, SOR, PHC, FLE	19	0.0035
all northern	CLF, HIC, LAI, MOR, HIS, SRI, JGR, LAK, CLI, FLE, PHC,	14	0.0033
MP-II	SOR, TEA, MUL, GSP		
all 27		18	0.0001
populations			

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-

proposed herein

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% between 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 within 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. kentuckensis 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_{sr} 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.

Acknowledgements

This research was supported by funds from TKP and JRJ, in addition to internal funding from Western Kentucky University through a Graduate Student Research Grant and a Research and Creative Activities Program (RCAP) grant. Naomi Rowland provided valuable advice and assistance with molecular work. We thank Dr. Matthew Niemiller for his general guidance and valuable comments on the manuscript, Dr. Karen Ober for her contribution of specimens, data, and advice, and Elise Valkanas for her phylogenetic studies on eastern North American cave beetles (supported by an NSF- REU grant) which inspired and contributed to this project. We thank Den and Sheila Roenfeldt for supporting a travel fellowship which allowed OFB to present this research and gather feedback from colleagues at the International Society for Subterranean Biology's 2016 conference. Our appreciation to Arnaud Faille and one anonymous reviewer whose insightful comments improved the manuscript. We thank the many landowners who granted permission for us to sample caves on private property, as well as Jim Currens, Julian Lewis, Ben Miller, Jason Polk, Lee Florea, Kurt Helf, The Rockcastle Karst Conservancy, the Green River Grotto, John Andersland, Rob Neidlinger, and the Kentucky Speleological Society.

References

- Barr TC (1967) Observations on the Ecology of Caves. The American Naturalist 101(922): 475–491. https://doi.org/10.1086/282512
- Barr TC (1969) Evolution of the (Coleoptera) Carabidae in the Southern Appalachians. In: Holt PC, Roane MK, Parker BC (Eds) The distributional history of the biota of the Southern Appalachians. Virginia Polytechnic Institute and State University, Blacksburg: 67–92.
- Barr TC (1971) Trechoblemus in North America, with a key to North American genera of Trechinae (Coleoptera: Carabidae). Psyche 78: 140–149. https://doi.org/10.1155/1971/14569
- Barr TC (1973) Refugees of the ice age. Natural History 82 5): 26-35, 72-73.
- Barr TC (1979) The taxonomy, distribution, and affinities of *Neaphaenops*, with notes on associated species of *Pseudanophthalmus* (Coleoptera, Carabidae). American Museum novitates: no. 2682: 1–20.
- Barr TC (1981) *Pseudanophthalmus* from Appalachian caves (Coleoptera: Carabidae): the engelhardti complex. Brimleyana 5: 37–94.
- Barr TC (1985) Pattern and process in speciation of trechine beetles in eastern North America (Coleoptera: Carabidae: Trechinae). In: Ball GE (Ed.) Taxonomy, Phylogeny and Zoogeography of Beetles and Ants. Dr W. Junk, Dordrecht: 350–407.
- Barr TC (2004) A classification and checklist of the genus *Pseudanophthalmus* Jeannel (Coleoptera: Carabidae: Trechinae). Virginia Museum of Natural History Special Publication 11: 1–52.
- Barr TC, Holsinger JR (1985) Speciation in Cave Faunas. Annual Review of Ecology and Systematics 16: 313–337. https://doi.org/10.1146/annurev.es.16.110185.001525
- Bousquet Y (2012) Catalogue of Geadephaga (Coleoptera, Adephaga) of America, north of Mexico. ZooKeys 245: 1–1722. https://doi.org/10.3897/zookeys.245.3416
- Caccone A (1985) Gene flow in cave arthropods: a qualitative and quantitative approach. Evolution 39(6):1223–1235. https://doi.org/10.1111/j.1558-5646.1985.tb05688.x
- Chessel D, Dufour AB, Thioulouse J (2004) The ade4 package I: One-table methods. R News 4: 5–10.
- Christman MC, Culver DC, Madden MK, White D (2005) Patterns of endemism of the eastern North American cave fauna. Journal of Biogeography 32(8): 1441–1452. https://doi. org/10.1111/j.1365-2699.2005.01263.x
- Christman MC, Doctor DH, Niemiller ML, Weary DJ, Young JA, Zigler KS, et al. (2016) Predicting the Occurrence of Cave-Inhabiting Fauna Based on Features of the Earth Surface Environment. PLoS ONE 11(8): e0160408. https://doi.org/10.1371/journal. pone.0160408
- Currens JC (2002) Kentucky is karst country! What you should know about sinkholes and springs. Information Circular 4, Series XII, University of Kentucky, Kentucky Geological Survey, 35 pp.
- Culver DC (1970) Analysis of Simple Cave Communities I. Caves as Islands. Evolution 24(2): 463–474. https://doi.org/10.1111/j.1558-5646.1970.tb01776.x
- Derkarabetian S, Steinmann DB, Hedin M (2010) Repeated and time-correlated morphological convergence in cave-dwelling harvestmen (Opiliones, Laniatores) from montane Western North America. PLoS One 5(5): e10388. https://doi.org/10.1371/journal.pone.0010388

- Diniz-Filho JA, Soares TN, Lima JS, Dobrovolski R, Landeiro VL, de Campos Telles MP, Rangel TF, Bini LM (2013) Mantel test in population genetics. Genetics and Molecular Biology 36(4): 475–85. https://doi.org/10.1590/S1415-47572013000400002
- Ersts PJ (2015) Geographic Distance Matrix Generator (version 1.2.3). American Museum of Natural History, Center for Biodiversity and Conservation.
- Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources 10: 564–567. https://doi.org/10.1111/j.1755-0998.2010.02847.x
- Faille A, Rene Tänzler R, Toussaint EFA (2015) On the way to speciation: Shedding light on the karstic phylogeography of the micro-endemic cave beetle *Aphaenops cerberus* in the Pyrenees. Journal of Heredity 106(6): 692–699. https://doi.org/10.1093/jhered/esv078
- Goudarzi F, Hemami M, Rancilhac L, et al. (2019) Geographic separation and genetic differentiation of populations are not coupled with niche differentiation in threatened Kaiser's spotted newt (*Neurergus kaiseri*). Scientific Reports 9: 6239. https://doi.org/10.1038/ s41598-019-41886-8
- Hanski I (1999) Metapopulation Ecology. Oxford University Press.
- Harker DF, Barr TC (1979) Caves and associated fauna of eastern Kentucky. Eastern Kentucky Coal Field: Preliminary Investigations of natural features and cultural resources. Vol. 3. Kentucky Nature Preserves Commission, Frankfort, 130 pp.
- Hebert PD, Ratnasingham S, de Waard JR (2003) Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proceedings of the Royal Society of London. Series B: Biological Sciences, 270(Suppl 1): S96–S99. https://doi.org/10.1098/ rsbl.2003.0025
- Hedin M, Thomas SM (2010) Molecular systematics of eastern North American Phalangodidae (Arachnida: Opiliones: Laniatores), demonstrating convergent morphological evolution in caves. Molecular Phylogenetics and Evolution 54: 107–121. https://doi.org/10.1016/j. ympev.2009.08.020
- Hobbs III HH (2012) Diversity patterns in the United States. In White WB, Culver DC (Eds) Encyclopedia of Caves. 2nd edition. Academic Press, Amsterdam, 251–264. https://doi. org/10.1016/B978-0-12-383832-2.00033-5
- Holsinger JR (1988) Troglobites: The Evolution of Cave-Dwelling Organisms. American Scientist 76(2): 146–153.
- Holsinger KE, Weir BS (2009) Genetics in geographically structured populations: defining, estimating and interpreting F(ST). Nature Reviews: Genetics 10: 639–650. https://doi.org/10.1038/nrg2611
- Huson DH, Bryant D (2006) Application of Phylogenetic Networks in Evolutionary Studies. Molecular Biology and Evolution 23(2): 254–267. https://doi.org/10.1093/molbev/msj030
- Kane TC, Barr TC, Badaracca WJ (1992) Cave beetle genetics: geology and gene flow. Heredity 68: 277–286. https://doi.org/10.1038/hdy.1992.40
- Katz AD, Taylor SJ, Davis MA (2018) At the confluence of vicariance and dispersal: Phylogeography of cavernicolous springtails (Collembola: Arrhopalitidae, Tomoceridae) codistributed across a geologically complex karst landscape in Illinois and Missouri. Ecology and Evolution 2018: 10306–10325. https://doi.org/10.1002/ece3.4507

- Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A (2012) Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28(12): 1647–1649. https://doi.org/10.1093/ bioinformatics/bts199
- Kentucky Geological Survey (2017) Kentucky Geologic Map Information Service, University of Kentucky.
- Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23(21): 2947–2948. https://doi.org/10.1093/ bioinformatics/btm404
- Lee CR, Mitchell-Olds T (2011) Quantifying effects of environmental and geographical factors on patterns of genetic differentiation. Molecular Ecology 20: 4631–4642 (2011). https:// doi.org/10.1111/j.1365-294X.2011.05310.x
- Lewis JJ, Lewis SL (2005) Cave fauna study for the Interstate 66 EIS (Somerset to London, Kentucky). Proceedings of the 2005 National Cave and Karst Management Symposium: 15–20.
- Maddison DR, Kanda K, Boyd OF, Faille A, Porch N, Erwin TL, Roig-Juñent S (2019) Phylogeny of the beetle supertribe Trechitae (Coleoptera: Carabidae): Unexpected clades, isolated lineages, and morphological convergence. Molecular Phylogenetics and Evolution 132: 151–176. https://doi.org/10.1016/j.ympev.2018.11.006
- Niemiller ML, Fitzpatrick BM, Miller BT (2008) Recent divergence with gene flow in Tennessee cave salamanders (Plethodontidae: *Gyrinophilus*) inferred from gene genealogies. Molecular Ecology 17 (9): 2258–2275. https://doi.org/10.1111/j.1365-294X.2008.03750.x
- Niemiller ML, Near TJ, Fitzpatrick BM (2012) Delimiting species using multilocus data: diagnosing cryptic diversity in the southern cavefish *Typhlichthys subterraneus* (Teleostei: Amblyopsidae). Evolution 66, 846–866. https://doi.org/10.1111/j.1558-5646.2011.01480.x
- Niemiller ML, McCandless JR, Reynolds RG, Caddle J, Tillquist CR, Near TJ, Pearson WD, Fitzpatrick BM (2013) Effects of climatic and geological processes during the Pleistocene on the evolutionary history of the northern cavefish, *Amblyopsis spelaea* (Teleostei: Amblyopsidae). Evolution 67: 1011–1025. https://doi.org/10.1111/evo.12017
- Niemiller ML, Zigler KS (2013) Patterns of cave biodiversity and endemism in the Appalachians and Interior Plateau of Tennessee, USA. PLoS ONE 8(5): e64177. https://doi. org/10.1371/journal.pone.0064177
- Peck SB (1998) A summary of diversity and distribution of the obligate cave-inhabiting faunas of the United States and Canada. Journal of Cave and Karst Studies 60(1): 18–26.
- Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P (1994) Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Annals of the Entomological Society of America 87(6): 651–701. https://doi.org/10.1093/aesa/87.6.651
- Teacher AGF, Griffiths DJ (2011) HapStar: automated haplotype network layout and visualization. Molecular Ecology Resources 11(1): 151–153. https://doi.org/10.1111/j.1755-0998.2010.02890.x

- Valentine JM (1952) New genera of anophthalmid beetles from cumberland caves (Carabidae, Trechini). Geological Survey of Alabama, Museum Paper 34, 41 pp.
- Villesen P (2007) FaBox: an online toolbox for fasta sequences. Molecular Ecology Notes 7(6): 965–968. https://doi.org/10.1111/j.1471-8286.2007.01821.x
- Wiens JJ, Chippindale PT, Hillis DM (2003) When are phylogenetic analyses misled by convergence? A case study in Texas cave salamanders. Systematic Biology 52: 501–514. https:// doi.org/10.1080/10635150309320
- Weir BS, Cockerham CC (1984) Estimating F-Statistics for the analysis of population structure. Evolution 38(6): 1358–1370. https://doi.org/10.1111/j.1558-5646.1984.tb05657.x
- White WB, Culver DC, Pipan T (2019) Encyclopedia of Caves, 3rd Edition, Academic Press, 1250 pp.

RESEARCH ARTICLE



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

I SNSB-Zoologische Staatssammlung, Münchhausenstrasse 21, D-81247 München, Germany **2** Institute of Evolutionary Biology (CSIC-Universitat Pompeu Fabra), Barcelona, Spain

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

Academic editor: Oana Moldovan | Received 15 January 2020 | Accepted 22 February 2020 | Published 10 March 2020

http://zoobank.org/E82D45EF-B6AF-4A17-831D-ED8D4DBD5A4E

Citation: Balke M, Ribera I (2020) A subterranean species of *Exocelina* diving beetle from the Malay Peninsula filling a 4,000 km distribution gap between Melanesia and southern China. Title. Subterranean Biology 34: 25–37. https://doi.org/10.3897/subtbiol.34.50148

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).

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.

Material examined. *Holotype male* (ZSM): Malaysia, Pahang, Cameron Highlands, Tanah Rata, Mount Berembun, 4.474705, 101.384043, 1,500m, 27.–29.ii.2012, K. Sugaya leg.

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 punctation. Elytra with distinct microreticulation formed by small regular cells and dense, coarse, setiferous punctation (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.

terior 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.



Figure 3. Ventral view of A Exocelina sugayai sp. nov. male and B Exocelina abdita.

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 metaxoca **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 &



Figure 5. *Exocelina sugayai* sp. nov. male genital, **A** median lobe of aedeagus in lateral view **B** paramere lateral inner view.

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).



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

- Balke M (1998) Revision of New Guinea Copelatus Erichson, 1832 (Insecta: Coleoptera: Dytiscidae): The running water species, Part I. Annalen des Naturhistorischen Museum Wien 100B: 301–341.
- Balke M (2001) Papuadessus pakdjoko a new genus and species of rheobiont diving beetle from New Guinea of potential use for environmental impact assessments (Coleoptera, Dytiscidae). Hydrobiologia 464(1–3): 107–112.
- Balke M, Bergsten J (2003) Copelatus (Papuadytes) shizong sp.n. from Yünnan (China), the first member of Papuadytes Balke found west of the Wallace Line. In: Jäch MA, Ji L (Eds) Water beetles of China. Vol. 3. Zoologisch- Botanische Gesellschaft in Österreich and Wiener Coleopterologenverein, Wien, 89–94.

- Balke M, Pons J, Ribera I, Sagata K, Vogler AP (2007) Infrequent and unidirectional colonization of hyperdiverse *Papuadytes* diving beetles in New Caledonia and New Guinea. Molecular Phylogenetics and Evolution 42: 505–516. https://doi.org/10.1016/j.ympev.2006.07.019
- Balke M, Watts CHS, Cooper SJB, Humphreys WF, Vogler AP (2004) A highly modified stygobiont diving beetle of the genus *Copelatus* (Coleoptera, Dytiscidae): taxonomy and cladistic analysis based on mitochondrial DNA sequences. Systematic Entomology 29: 59–67. https://doi.org/10.1111/j.1365-3113.2004.00229.x
- Caetano DS, Bená DD, Vanin SA (2013) *Copelatus cessaima* sp. nov. (Coleoptera: Dytiscidae: Copelatinae): first record of a troglomorphic diving beetle from Brazil. Zootaxa 3710(3): 226–232. https://doi.org/10.11646/zootaxa.3710.3.2
- Kalyaanamoorthy S, Minh BQ, Wong TKF, von Haeseler A, Jermiin LS (2017) ModelFinder: Fast model selection for accurate phylogenetic estimates. Nature Methods 14: 587–589. https://doi.org/10.1038/nmeth.4285
- Kearse M, Moir R, Wilson A, Stones-Hava S, Cheung, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A (2012) Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28: 1647–1649. https://doi.org/10.1093/bioinformatics/bts199
- Lam AW, Gueuning M, Kindler C, Van Dam M, Alvarez N, Panjaitan R, Shaverdo H, White LT, Roderick GK, Balke M (2018) Phylogeography and population genomics of a lotic water beetle across a complex tropical landscape. Molecular Ecology 27: 3346–3356. https:// doi.org/10.1111/mec.14796
- Miller KB, Bergsten J (2016) Diving beetles of the world. Systematics and biology of the Dytiscidae. Baltimore: Johns Hopkins University Press, 320 pp.
- Nguyen L-T, Schmidt HA, von Haeseler A, Minh BQ (2015) IQ- TREE: A fast and effective stochastic algorithm for estimating maximum likelihood phylogenies. Molecular Biology and Evolution 32(1): 268–274. https://doi.org/10.1093/molbev/msu300
- Nilsson AN, Hájek J (2019) A world catalogue of the family Dytiscidae (Coleoptera, Adephaga). 307 pp. [Internet version 1.I.2019] http://www.waterbeetles.eu
- Shaverdo H, Balke M (2019) A new species of the *Exocelina ekari* group and new faunistic data on 12 species of *Exocelina* Broun, 1886 from New Guinea (Coleoptera: Dytiscidae). Koleopterologische Rundschau 89: 1–10.
- Shaverdo H, Sagata K, Balke M (2018) Introduction of the *Exocelina casuarina*-group, with a key to its representatives and descriptions of 19 new species from New Guinea (Coleoptera, Dytiscidae, Copelatinae). ZooKeys 803: 7–70. https://doi.org/10.3897/zookeys.803.28903
- Shaverdo HV, Surbakti S, Hendrich L, Balke M (2012) Introduction of the *Exocelina ekari*group with descriptions of 22 new species from New Guinea (Coleoptera, Dytiscidae, Copelatinae). ZooKeys 250: 1–76. https://doi.org/10.3897/zookeys.250.3715
- Shaverdo HV, Surbakti, Warikar EL, Sagata K, Balke M (2019) Nine new species groups, 15 new species, and one new subspecies of New Guinea diving beetles of the genus *Exocelina* Broun, 1886 (Coleoptera, Dytiscidae, Copelatinae). ZooKeys 878: 73–143. https://doi. org/10.3897/zookeys.878.37403
- Toussaint EFA, Hall R, Monaghan MT, Sagata K, Ibalim S, Shaverdo HV, Vogler AP, Pons J, Balke M (2014) The towering orogeny of New Guinea as a trigger for arthropod megadiversity. Nature Communications 5: 4001. https://doi.org/10.1038/ncomms5001
- Toussaint EFA, Henrich L, Shaverdo H, Balke M (2015) Mosaic patterns of diversification dynamics following the colonization of Melanesian islands. Scientific Reports 5: 16016. https://doi.org/10.1038/srep16016
- Villastrigo A, Fery H, Manuel M, Millán A, Ribera I (2018) Evolution of salinity tolerance in the diving beetle tribe Hygrotini (Coleoptera, Dytiscidae). Zoologica Scripta 47: 63–71. https://doi.org/10.1111/zsc.12255
- Watts CHS, Humphreys WF (2009) Fourteen new Dytiscidae (Coleoptera) of the genera Limbodessus Guignot, Paroster Sharp, and Exocelina Broun from underground waters in Australia. Transactions of the Royal Society of South Australia 133(1): 62–107. https://doi.org/10.1080/03721426.2009.10887112
- Watts CHS, Hendrich L, Balke M (2016) A new interstitial species of diving beetle from tropical northern Australia provides a scenario for the transition of epigean to stygobitic life (Coleoptera, Dytiscidae, Copelatinae). Subterranean Biology 19: 23–29. https://doi. org/10.3897/subtbiol.19.9513

Subterranean Biology 34: 39–59 (2020) doi: 10.3897/subtbiol.34.50231 http://subtbiol.pensoft.net

RESEARCH ARTICLE



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

Alberto Sendra¹, Heriberto López², Jesús Selfa³, Pedro Oromí⁴

I Grupo de Investigación de Biología del Suelo y de los Ecosistemas Subterráneos, Departamento de Ciencias de la Vida, Facultad de Biología, Ciencias Ambientales y Química, Universidad de Alcalá, E-28871 - Alcalá de Henares, Madrid, Spain 2 Island Ecology and Evolution Research Group (GEEI), Instituto de Productos Naturales y Agrobiología (IPNA-CSIC), 38206 La Laguna, Tenerife, Canary Islands, Spain 3 Departament de Zoologia. Laboratori d'Investigació d'Entomologia. Universitat de València. Carrer Dr. Moliner s/n. 46100 Burjassot, València, Spain 4 Depto. Biología Animal, Universidad de La Laguna, 38206 La Laguna, Tenerife, Canary Islands, Spain

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

Academic editor: O. T. Moldovan Received 17 January 2020 Accepted 22 February 2020 Publish	ied 18 March 2020

Citation: Sendra A, López H, Selfa J, Oromí P (2020) Two new dipluran species unearthed from subterranean habitats of the Canary Islands (Arthropoda, Hexapoda, Entognatha). Subterranean Biology 34: 39–59. https://doi.org/10.3897/subtbiol.34.50231

Abstract

Two new dipluran species of the family Campodeidae have been unearthed in the Canary Islands. *Remy-campa 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.

Copyright Alberto Sendra et al. This is an open access article distributed under the terms of the Creative Commons Attribution License (CC BY 4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

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; Racovitz 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 lightperceptive 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



Figures 1–3. Cueva de Montaña Blanca, El Castillo, Fuerteventura, Canary Islands. I Interior of the volcanic tube **2** entrance to the cave through a concrete tunnel **3** view of the entrance of the cave in an abandoned building.

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-



Figure 4. Brezal del Palmital, Gran Canaria, Canary Islands, Spain, site in the MSS where a pitfall was installed; dashed line shows the limit between epigean and hypogean (i.e. subterranean) environments.

nae according to Bareth and Condé (1981). For the position of macrosetae we adopt the abbreviations of Condé (1955): *ma*, medial-anterior *la*, lateral-anterior, *lp*, lateral-posterior and *post*, posterior.

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 μ l total PCR volume containing 15.4 μ l of purified water, 2.5 μ l of 10x NH₄-based Reaction Buffer, 1.5 μ l of MgCl₂ (3mM), 2 μ l of 10 mM dNTP (2.5 mM each), 0.5 μ l of BSA, 1 μ l of each primer (10 μ M), 0.1 μ l of BIOTAQTM DNA polymerase, and 1 μ l 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 \bigcirc , 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 $\bigcirc \bigcirc$, 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 $\bigcirc \bigcirc$), IPNA-CSIC (1 \bigcirc), ASM (2 $\bigcirc \bigcirc$, 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 $2\times$ 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 μ m 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 a/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).

Specimen	Body	Antennae	Number of	Metathoracic leg					
	length	length	antennomeres	Coxa	Trochanter	Femur	Tibia	Tarsus	Total leg
Paratype, 👌 1	4.4	-	-	0.16	0.12	0.60	0.76	0.53	2.17
Holotype, \bigcirc	4.2	3.54	36	0.18	0.15	0.62	0.72	0.54	2.21
Paratype, 👌 2	4.0	-	-	0.18	0.12	0.52	0.80	0.50	2.12
Paratype, ∂5	3.9	_	-	0.16	0.10	0.53	0.74	0.51	2.04
Paratype, ♂3	3.8	_	-	0.15	0.10	0.51	0.70	0.49	1.95
Paratype, J	2.2	_	_	0.10	0.08	0.36	0.38	0.30	0.92

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



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.



Figures 17–20. *Remycampa herbanica* sp. nov. 17 Pronotum 18 detail of pronotum with medial anterior macrosetae 19 detail of pronotum with lateral anterior and lateral posterior macrosetae 20 detail of pronotum with clothing setae.

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, meta-thoracic legs reaching abdominal segment IX, about 0.5× 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*₃ on V–VII; 1+1 *mp*, 3+3 *lp*₁₋₃ on VIII; and 1+1 *mp*, 5+5 *lp*₁₋₅ on IX abdominal segment. All tergal abdominal macrosetae short, slightly thick with thin short barbs being *ma* and *mp* the shortest.



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



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).

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.

							Cer	rci, arti	cles ler	igth					
Divisions basal	Basal	1 st	2 nd	3 ^{tr}	4^{th}	5 th	6 th	7 th	8 th	9 th	10^{th}	11 th	12 th	13 th	14^{th}
article															
10	1.25	0.16	0.18	0.20	0.19	0.19	0.20	0.23	0.22	0.24	0.26	0.26	0.25	0.26	0.28
10	1.58	0.18	0.18	0.18	0.18	0.21	0.22	0.23	0.24	0.25	0.28	0.27	0.26	0.27	0.29
Divisions basal	15 th	16 th	17^{th}	18 th	19 th	20^{th}	21 th	22 th	23 th	24^{th}	25 th	26 th	27^{th}	Total	cercus
article															
10	0.28	0.30	0.30	0.30	0.32	0.32	0.30	0.34	0.32	0.34	0.35	0.33	0.30	8.	66
10	0.28	0.29	0.29	0.31	0.31	0.33	_	-	-	-	-	-	-	-	_

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

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

Male urosternite I with short coniform appendages, each bearing about 13 glandular a_1 setae in a distal field; posterior edge occupied by a large but narrow field of cramped up to 190 glandular g_1 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 \bigcirc , 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 \bigcirc , 1 \bigcirc (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 $12 \mu m$ 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 a_1 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 a_1 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 g_1 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 ursoternite, 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 3+3 lateral posterior on 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 (Monocampa) 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 species 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.

Acknowledgements

We are extremely grateful to Bernardo Rodríguez and Sofía Menéndez for his help with sampling efforts; and for SEM facilities provided by the Universitat de València, with special thanks to Enrique Navarro, Pilar Gómez and Rafael Benito. We also thank Guido Jones and Katie Marsen for helping us to translate this paper.

References

Bareth C, Condé B (1981) Nouveaux Campodéidés de grottes d'Espagne. Revue suisse Zoologie 88(3): 775–786. https://doi.org/10.5962/bhl.part.82407

- Borges P, Oromí P (1994) Azores. In: Juberthie C, Decu V (Eds) Encyclopaedia Biospeologica, vol. I. Société de Biospéologie, Moulis-Bucarest: 605–610.
- Condé B (1953) Campodéidés endogés d'Afrique septentrionale. Bulletin de la Société Zoologique de France 78(5–6): 358–377.
- Condé B (1955) Matériaux pour une monographie des Diploures Campodéidés. Mémoires du Muséum national d'histoire naturelle, série A, Zoologie 12: 1–202.
- Culver DC, Pipan T (2014) Shallow subterranean habitats: Ecology, evolution and conservation. Oxford University Press, 288 pp. https://doi.org/10.1093/acprof:oso/9780199646173.001.0001
- Culver DC, Pipan T (2010) The biology of caves and other subterranean habitats. Oxford University Press, 254 pp.
- Deharveng L, Bedos A (2018) Diversity of Terrestrial Invertebrates in Subterranean Habitats. In: Moldovan OT, Kováč L, Halse S (Eds) Cave Ecology. Springer, Switzerland, 107–172. https://doi.org/10.1007/978-3-319-98852-8_7
- Ferguson LM (1992) Diplura of lava tube caves. Proceedings of the 6th International Symposium Vulcacospeleology, Hilo Hawaii, 281–284.
- Fernández O, Naranjo M, Martín S (2015) Cueva de Aslobas: hallazgo del tubo volcánico más antiguo de las Islas Canarias. 1ª Convenció Internacional d'Espeleologia, Barcelona, 75–82. https://www.researchgate.net/publication/313267622_Cueva_de_Aslobas_hallazgo_del_tubo_volcanico_mas_antiguo_de_las_islas_Canarias
- Fernández-Palacios JM, De Nascimento L, Otto R, Delgado JD, García-Del-Rey E, Arévalo JR, Whittaker RJ (2011) A reconstruction of Palaeo-Macaronesia, with particular reference to the long-term biogeography of the Atlantic island laurel forests. Journal of Biogeography 38: 226–246. https://doi.org/10.1111/j.1365-2699.2010.02427.x
- Fernández-Palacios JM, Rijsdijk kf, Norder SJ, Otto R, De Nascimento L, Fernández-Lugo S, Tjørve E, Whittaker RJ (2015) Towards a glacial-sensitive model of island biogeography. Global Ecology and Biogeography 25(7): 817–830. https://doi.org/10.1111/geb.12320
- Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3: 294–299. https://www.researchgate.net/publication/15316743_DNA_primers_for_amplification_of_mitochondrial_Cytochrome_C_ oxidase_subunit_I_from_diverse_metazoan_invertebrates
- George M (1963) Studies on *Campodea* (Diplura) the anatomy of the glands and sense-organs of the head. Journal of Cell Science s3-104: 1–21. https://jcs.biologists.org/content/s3-104/65/1
- Hebert PDN, Cywinska A, Ball SL, Dewaard JR (2003) Biological identifications through DNA barcodes. Proceedings of the Royal Society of London B 270: 313–321. https:// royalsocietypublishing.org/doi/10.1098/rspb.2002.2218
- Howarth FG (2008) Caves. In: Faith BD (Ed.) Encyclopaedia of ecology. Elsevier, 536–541. https://doi.org/10.1016/B978-008045405-4.00712-6
- Juberthie C, Delay B, Bouillon M (1980) Extension du milieu souterrain en zone non-calcaire: description d'un nouveau milieu et de son peuplement par des coléoptères troglobies. Mémoires de Biospéologie 7: 19–52.

- Katoh K, Misawa K, Kuma K, Miyata T (2002) MAFFT: A novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Research 30(14): 3059–3066. https://doi.org/10.1093/nar/gkf436
- Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Mentjies P, Drummond A (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28(12): 1647–1649. https://doi.org/10.1093/ bioinformatics/bts199
- López H, Oromí P (2010) A pitfall trap for sampling the mesovoid shallow substratum (MSS) fauna. Speleobiology Notes 2: 7–11. http://www.nsm.buffalo.edu/Research/SPELEOBI-OLOGY_NOTES/index.php/Speleo/article/view/19
- Mammola S, Giachino PM, Piano E, Jones A, Barberis M, Badino G, Isaia M (2016) Ecology and sampling techniques of an understudied subterranean habitat: the Milieu Souterrain Superficiel (MSS). The Science of Nature 103(11–12): 1–24. https://doi.org/10.1007/ s00114-016-1413-9
- Medina AL, Oromí P (1990) First data on the superficial underground compartment on La Gomera (Canary Islands). Mémoires de Biospéologie 17: 87–91.
- Molero R, Gaju M, López N, Oromí P, Bach C (2013) The family Nicoletiidae in the Canary Islands, with description of new taxa (Insecta: Zygentoma). European Journal of Entomology 111(2): 267–274. https://doi.org/10.14411/eje.2014.032
- Naranjo M, Oromí P (2011) La cueva de Montaña Blanca: nuevo tubo volcánico en la isla de Fuerteventura. Vulcania 9: 49–51. https://mdc.ulpgc.es/cdm/ref/collection/vul/id/77
- Oromí P, Martín JL (1992) The Canary Islands. Subterranean fauna, characterization and composition. In: Camacho AI (Ed.) The natural history of biospeleology. C.S.I.C., Madrid, 527–567.
- Oromí P, Medina AL, Tejedor ML (1986) On the existence of a superficial underground compartment in the Canary Islands. Actas IX Congreso Internacional de Espeleología. Barcelona 2: 147–151.
- Ortuño VM, Gilgado JD, Jiménez-Valverde A, Sendra A, Pérez-Suárez G, Herrero-Borgoñón JJ (2013) The "Aluvial Mesovoid Shallow Substratum", a new subterranean habitat. PLoS ONE 8 (10): e76311. https://doi.org/10.1371/journal.pone.0076311
- Paclt J (1957) Diplura. In: Wytsman P (Ed.) Genera Insectorum, 212° fasc: 1–123.
- Paclt J, Báez M (1990) Présence de Diploures Iapygidés dans les Îles Canaries (Insecta, Diplura, Iapygidae). Vieraea 18: 121–122. https://www.museosdetenerife.org/assets/downloads/ publication-ca6a0df61d.pdf
- Paclt J, Báez M (1992) Sur une seconde espèce de Diploures Iapygidés (Insecta) des Îles Canaries. Bocagiana 161: 1–2. http://www.ibigbiology.com/fotos/publicacoes/publicacoes_Paclt92_SurSecondeEspeceDiplouresLapygidesllesCanaries.pdf
- Pagés J (1959) Remarques sur la classification des diploures. Travaux du Laboratoire de Zoologie et de la Station Aquicole Grimaldi de la Faculté des Sciences de Dijon 26: 1–25.
- Pagés J (1989) Sclérites et appendices de l'abdomen des Diploures (Insecta, Apterygota). Archives des Sciences Genève 42(3): 509–551.
- Pagés J (1993) Japygidés d'Europe et du bassin méditerranéen n° 8 -Dicellurata Genavensia XVIII. Revue suisse de Zoologie 100(1): 47–64. https://doi.org/10.5962/bhl.part.82499

- Pipan T, López H, Oromí P, Polack S, Culver DC (2010) Temperature variation and the presence of troglobionts in terrestrial shallow subterranean habitats. Journal of Natural History 45(3–4): 253–273. https://doi.org/10.1080/00222933.2010.523797
- Price MN, Dehal PS, Arkin AP (2009) FastTree: Computing Large Minimum-Evolution Trees with Profi les instead of a Distance Matrix. Molecular Biology and Evolution 26: 1641– 1650. https://doi.org/10.1093/molbev/msp077
- Racovitză EG (1907) Essai sur les problèmes biospéologiques. Archives de Zoologie Expérimentale et Générale 6: 371–488.
- Rambla M (1993) Maiorerus randoi n. gen., n. sp., the first laniatorid from a Canary Island cave (Opiliones, Phalangodidae). Mémoires de Biospéologie 20: 177–182.
- Rando JC, Sala L, Oromí P (1993) The hypogean community of Cueva del Llano (Fuerteventura, Canary Islands). Mémoires de Biospéologie 20: 189–193.
- Rusek J (1982) Octostigma herbívora n. gen. & sp. (Diplura: Projapygidae: Octostigmatidae n. fam.) injuring plant roots in the Tonga Islands. New Zealand Journal Zoology 9: 25–32. https://doi.org/10.1080/03014223.1982.10423833
- Sendra A (1989) Datos sobre Campodeidos del Museo Nacional de Ciencias Naturales de Madrid (Insecta, Diplura, Campodeidae). Eos 65(1): 115–123. http://digital.csic.es/handle/10261/172646
- Sendra A (1990) Campodeidos del Archipiélago Canario (Insecta, Diplura). Actas IV Congreso Ibérico de Entomologia, X Jornadas de la AeE, Sant Feliu de Guíxols (Gerona): 100.
- Sendra A (2015) Orden Diplura. Ibero Diversidad Entomológica @ccesible-SEA 35: 1–11. http://sea-entomologia.org/IDE@/revista_35.pdf
- Sendra A, Antić D, Barranco P, Borko Š, Christian E, Delić T, Fadrique F, Faille A, Galli L, Gasparo F, Georgiev D, Giachino PM, Kováč L, Lukić M, Marcia P, Miculinić K, Nicolosi G, Palero F, Paragamian K, Pérez T, Polak S, Prieto CE, Turbanov I, Vailati D, Reboleira ASPS (2020) Flourishing in subterranean ecosystems: Euro-Mediterranean Plusiocampinae and tachycampoids (Diplura, Campodeidae). European Journal of Taxonomy 591: 1–138. https://doi.org/10.5852/ejt.2020.591
- Sendra A, Báez M (1986) Nota sobre los Dipluros Campodeidos de la isla de Tenerife (Islas Canarias) (Diplura Campodeidae). Fragmenta Entomologica 19(1): 95–98.
- Sendra A, Jiménez A, Rochat J, Legros V, Gasnier S, Cazanove G (2017) A new and remarkable troglobitic *Lepidocampa* Oudemans, 1890 species from La Réunion island, with a discussion on troglobiomorphic adaptations in campodeids (Diplura). Zoologischer Anzeiger 266: 95–104. https://doi.org/10.1016/j.jcz.2016.11.005
- Sendra A, Palacios J, García A, Montejo M (2016) New species of Campodeidae (Diplura) from Mexican caves. Zootaxa 4072(5): 540–558. https://doi.org/10.11646/zootaxa.4072.5.2
- Silvestri F (1933) Primo contributo alla conoscenza dei Campodeidae dell'Africa Occidentale. Bolletino del Laboratorio di Zoologia generale e agraria in Portici 27: 205–218.
- Wood C (1979) Lava tubes: their morphogenesis and role in flow formation. The Cascade Caver (Seattle) 18(5–6): 27–30.
- Wood C, Mills M (1977) Geology of the lava tube caves around Icod de los Vinos, Tenerife. Transactions British Cave Research Association 4(4): 453–469.

Subterranean Biology 34:61–98 (2020) doi: 10.3897/subtbiol.34.49586 http://subtbiol.pensoft.net

RESEARCH ARTICLE



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

I 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)

Academic editor: O. Moldovan	Received 23 December 2019 Accepted 22 February 2020	Published 26 March 2020
http:/	//zoobank.org/3FD733A3-F576-42AC-9ACA-7CAF915DD82C	

Citation: Feng Z, Wynne JJ, Zhang F (2020) Cave-dwelling pseudoscorpions of China with descriptions of four new hypogean species of *Parobisium* (Pseudoscorpiones, Neobisiidae) from Guizhou Province. Subterranean Biology 34: 61–98. https://doi.org/10.3897/subtbiol.34.49586

Abstract

We summarize and discuss the 29 known cave-dwelling pseudoscorpion species from China. Four new troglomorphic 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 (Morikawa 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

caves originally sampled by M. Tian. 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 I. 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 troglophiles. 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). Troglophiles (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-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.

Taxa	Category	#Cave	Province	Reference
Family Chernetidae				
Megachernes glandulosus Mahnert, 2009	Troglophile	1	Hubei	Mahnert (2009)
Megachernes himalayensis (Ellingsen, 1914)	Troglophile	1	Guangxi	Schawaller (1995)
Megachernes tuberosus Mahnert, 2009	Troglophile	1	Sichuan	Mahnert (2009)
Megachernes vietnamensis Beier, 1967	Troglophile	3	Sichuan, Hubei	Schawaller (1995)
Nudochernes lipase Mahnert, 2003	Troglophile	1	Yunnan	Mahnert (2003)
Nudochernes troglobius Mahnert, 2009	Troglophile	2	Hubei, Sichuan	Mahnert (2009)
Family Chthoniidae				
Lagynochthonius bailongtanensis Li, Liu & Shi, 2019	Troglobiont	1	Yunnan	Li et al. (2019)
Tyrannochthonius akaleus Mahnert, 2009	Troglobiont	1	Sichuan	Mahnert (2009)
Tyrannochthonius antridraconis Mahnert, 2009	Troglobiont	4	Sichuan	Mahnert (2009)
Tyrannochthonius chixingi Gao, Wynne & Zhang, 2018	Troglobiont	1	Guangxi	Gao et al. (2018)
Tyrannochthonius ganshuanensis Mahnert, 2009	Troglobiont	3	Sichuan, Hubei	Mahnert (2009)
Family Neobisiidae				
Bisetocreagris baozinensis Mahnert & Li, 2016	Troglobiont	1	Sichuan	Mahnert and Li (2016)
Bisetocreagris cavernarum Mahnert & Li, 2016	Troglobiont	1	Chongqing	Mahnert and Li (2016)
Bisetocreagris chinacavernicola (Schawaller, 1995)	Troglobiont	2	Sichuan	Schawaller (1995)
Bisetocreagris chuanensis Mahnert & Li, 2016	Troglobiont	2	Guizhou	Mahnert and Li (2016)
Bisetocreagris gracilenta Gao & Zhang, 2017	Troglobiont	1	Guizhou	Gao et al. (2017)
Bisetocreagris guangshanensis Gao & Zhang, 2017	Troglobiont	1	Guizhou	Gao et al. (2017)
Bisetocreagris juanxuae Mahnert & Li, 2016	Troglobiont	1	Sichuan	Mahnert and Li (2016)
Bisetocreagris maomaotou Gao, Wynne & Zhang, 2018	Troglobiont	1	Guangxi	Gao et al. (2018)
Bisetocreagris martii (Mahnert, 2003)	Troglobiont?	1	Yunnan	Mahnert (2003)
Bisetocreagris scaurum (Mahnert, 2003)	Troglobiont	1	Yunnan	Mahnert (2003)
Bisetocreagris titanium (Mahnert, 2003)	Troglobiont	1	Yunnan	Mahnert (2003)
Bisetocreagris xiaoensis Li & Liu, 2017	Troglobiont	1	Yunnan	Li et al. (2017)
Parobisium magangensis Feng, Wynne & Zhang, 2019	Troglobiont	1	Beijing	Feng et al. (2019)
Parobisium motianense sp. nov.	Troglobiont	1	Guizhou	This study
Parobisium qiangzhuang sp. nov.	Troglobiont	1	Guizhou	This study
Parobisium sanlouense sp. nov.	Troglobiont	1	Guizhou	This study
Parobisium tiani sp. nov.	Troglobiont	1	Guizhou	This study
Parobisium yuantongi Feng, Wynne & Zhang, 2019	Troglobiont	1	Beijing	Feng et al. (2019)

Results

Family Neobisiidae Chamberlin, 1930 Subfamily Neobisiinae Chamberlin 1930

Genus Parobisium Chamberlin 1930

- *Neobisium (Parobisium)* Chamberlin 1930: 17; Beier 1932: 84; Morikawa 1960: 112–113; Hoff 1961: 427.
- *Parobisium* Chamberlin: Chamberlin and Malcolm 1960: 112–113; Chamberlin 1962: 123; Harvey 1991: 394; Mahnert 2003: 744–745.

Type species. Neobisium (Parobisium) magnum Chamberlin, 1930, by original designation.

Key to Parobisium species of China

1	Carapace with eyes or eye spots
_	Carapace without eyes or eye spots
2	Carapace only with two eyes or eye spots
_	Carapace with four eves or evespots
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	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
5	Carapace with four developed eyes; epistome small, triangular; pedipalp without
	granulation
_	Carapace with four eyespots; epistome small, rounded; pedipalp with finely gran-
	ulation
6	Pedipalpal femur 6.50–6.59 times longer than wide, patella 5.07–5.11 times lon-
	ger than wide
_	Boyh pedipalpal femur and patella less than 5.0 times longer than wide7
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 &



Figure 2. Parobisium motianense sp. nov. Sex indeterminable from photo.

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. 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); 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. tiani; 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. tiani; 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. tiani; 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. wangae*; 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



Figure 4. *Parobisium motianense* sp. nov., holotype male (**A**–**F**, **H**–**I**), female (**G**). **A** Carapace, dorsal view **B** Eye area, lateral view **C** Left chelicera, dorsal view **D** Right chela, lateral view **E** Right pedipalp, dorsal view **F** Male genitalia **G** Female genitalia **H** Left leg I, lateral view **I** Left leg IV, lateral view.

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 *et*; 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.

Abdomen: Pleural membrane granulated. Tergal chaetotaxy (I–XI): 8–11/9– 10/9/9–11/10–11/10–11/9–12/11–12/11–12/10–11/6–8; sternal chaetotaxy (IV– 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.

Legs: Coxa chaetotaxy (I–IV): 8-9/6-7/3-4/9-10. Leg I (Figs 4H, 5K): femur 4.37–4.96, patella 2.92–3.22, tibia 6.00–7.27, basitarsus 3.33–3.85, telotarsus 5.50–5.57 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 slender 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 transparent sclerotic 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): 8/7/9/10/10/11/11/11/11/10/6; sternal chaetotaxy (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 parentheses). 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, movable 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 zhuàng (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



Figure 7. Parobisium qiangzhuang sp. nov. A Holotype male, dorsal view B Paratype female, dorsal view.

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): 10–11/11/11– 12/12/12–13/12/12/12/12/12–13/12/7–9; 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 (with 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



Figure 10. Parobisium sanlouense sp. nov. Male habitus.

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. xiaowutai-cum*); 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. 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. 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. xiaowutaicum*; 5.70 times in *P. qiangzhuang*; 4.97–5.03 times in *P. tiani*; 3.13–3.52 times in *P. angangensis*; 3.72–4.06 times in *P. wangae*; 3.14 times in *P. xiaowutaicum*; 5.70 times in *P. tiani*; 3.13–3.52 times in *P. wangae*; 3.14 times in *P. xiaowutaicum*; 5.70 times in *P. tiani*; 3.13–3.52 times in *P. wangae*; 3.14 times in *P. xiaowutaicum*; 5.70 times in *P. motianense*; 69–80 in *P. qiangzhuang*; 57–74 in *P. wangae*; 73–75 in *P. xiaowutaicum*).

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



Figure 11. Parobisium sanlouense sp. nov. A Holotype male, dorsal view B Paratype female, dorsal view.

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 nearer *b* than to *st* (Figs 12D, 13H, J). Venom apparatus present only in fixed chelal finger with 126–130 teeth, movable finger with 119–126 teeth.

Abdomen: Pleural membrane granulated. Tergal chaetotaxy (I–XI): 7/7–9/9– 10/10/8–10/11/11/11/11–12/11/7; sternal chaetotaxy (IV–XI): 9–10/13/12/12–13/10– 13/13–14/12/3; stigmata with 4–6 setae; anal cone with 2 dorsal and 2 ventral setae. Male genital area (Figs 12F, 13D): sternite II with 34–36 scattered setae; sternite III with anteromedian groove flanked by one small seta on each side, with 21–30 posterior setae.

Legs: Coxa chaetotaxy (I–IV): 8-9/5-6/5/9. Leg I (Figs 12H, 13L): femur 5.08– 5.73, patella 3.82–4.05, tibia 8.21–8.57, basitarsus 4.00–4.33, telotarsus 6.08–6.58 times longer than deep, femur 1.45–1.48 times longer than patella, telotarsus 1.40–



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.

Pedipalps. Pedipalpal coxa with 8–9 setae. Trochanter 2.59–2.89, femur 6.03–6.60, patella 4.52–4.72, chela (with pedicel) 5.07–5.35, chela (without pedicel) 4.52–4.77 times longer than wide, movable finger 2.00–2.02 times longer than hand (without pedicel). Fixed chelal finger with 119–128 teeth, movable finger with 115–125 teeth.

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. giangzhuang 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 pedipalps 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. Parobisium tiani 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, *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 *et*; on movable finger, *st* nearer to *t* than to *sb*, the latter slightly nearer *st* than to *b* (Figs 16F, 17H–I). Venom apparatus present only in fixed chelal finger, wenom duct short, not extending past half of the distance to *et*. Fixed chelal finger with 104–109 teeth, movable finger with 105–112 teeth.

Abdomen: Pleural membrane granulated. Tergal chaetotaxy (I–XI): 8–9/ 8–9/ 9/ 10/ 9/ 9–11/ 9–10/ 10–11/ 10–12/ 10–11/ 6–7; sternal chaetotaxy (IV–XI): 7–8/ 12–13/ 14/ 12–13/ 11–13/ 11–12/ 11–12/ 3–4; stigmata with 5–6 setae; anal cone with 2 dorsal and 2 ventral setae. Male genital area (Figs 16G, 17F): sternite II with



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.

Abdomen. Tergal chaetotaxy (I–XI): 6–7/ 7–9/ 8–9/ 8–10/ 9–11/ 10–12/ 9–12/ 9–12/ 11–12/ 11/ 6–7; sternal chaetotaxy (IV–XI): 8–9/ 13–15/ 13–16/ 13–15/ 13–



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, H–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 galea 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



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-



Figure 20. Sanlou Cave, type locality of *Parobisium sanlouense* sp. nov. **A** Surrounding surface vegetation with cave entrance (white arrow) and sand mining operations adjacent to the cave **B** Entrance **C** Area where *P. sanlouense* specimens were collected **D** Terminus of Sanlou Cave with the potable water reservoir; specimens were collected near the reservoir.

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.



Figure 21. Biyun Cave, type locality of *Parobisium tiani* sp. nov. **A** Surrounding surface habitat with two entrances, entrance 1 (white arrow) and entrance 2 (red arrow) **B** Entrance 1 (white arrow) **C** Entrance 2 (red arrow) **D** Passage where specimens were collected (blue arrow).

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

- Barr Jr TC (1967) Observations on the ecology of caves. American Naturalist 101: 475–491. https://doi.org/10.1086/282512
- Beier M (1932) Pseudoscorpionidea I. Subord. Chthoniinea et Neobisiinea. Tierreich 57: 1–258. https://doi.org/10.1515/9783111435107
- Borges PAV, Cardoso P, Amorim IR, Pereira F, Constância JP, Nunes JC, Barcelos P, Costa P, Gabriel R, Dapkevicius ML (2012) Volcanic caves: priorities for conserving the Azorean endemic troglobiont species. International Journal of Speleology 41: 101–112. https://doi. org/10.5038/1827-806X.41.1.11
- Brooks TM, Pimm SL, Oyugi JO (1999) Time lag between deforestation and bird extinction in tropical forest fragments. Conservation Biology 13: 1140–1150. https://doi.org/10.1046/ j.1523-1739.1999.98341.x
- Castaño-Sánchez A, Hose GC, Reboleira ASPS (2019) Ecotoxicological effects of anthropogenic stressors in subterranean organisms: A review. Chemosphere 125422. https://doi. org/10.1016/j.chemosphere.2019.125422
- Chamberlin JC (1930) A synoptic classification of the false scorpions or chela-spinners, with a report on a cosmopolitan collection of the same. Part II. The Diplosphyronida (Arachnida-Chelonethida). Annals and Magazine of Natural History (10) 5: 1–48. https://doi.org/10.1080/00222933008673104
- Chamberlin JC (1931) The arachnid order Chelonethida. Stanford University Publications. Biological Sciences 7: 1–284.
- Chamberlin JC (1962) New and little-known false scorpions, principally from caves, belonging to the families Chthoniidae and Neobisiidae (Arachnida, Chelonethida). Bulletin of the American Museum of Natural History 123: 303–352.
- Chamberlin JC, Malcolm DR (1960) The occurrence of false scorpions in caves with special reference to cavernicolous adaptation and to cave species in the North American fauna (Arachnida-Chelonethida). American Midland Naturalist 64: 105–115. https://doi. org/10.2307/2422895
- Chevaldonné P, Lejeune C (2003) Regional warming-induced species shift in north-west Mediterranean marine caves. Ecology Letters 6: 371–379. https://doi.org/10.1046/j.1461-0248.2003.00439.x
- Christman MC, Culver DC, Madden MK, White D (2005) Patterns of endemism of the eastern North American cave fauna. Journal of Biogeography 32: 1442–1452. https://doi. org/10.1111/j.1365-2699.2005.01263.x
- Clements R, Sodhi NS, Schilthuizen M, Ng PK (2006) Limestone karsts of Southeast Asia: imperiled arks of biodiversity. Bioscience 56: 733–742. https://doi.org/10.1641/0006-3568(2006)56%5B733:LKOSAI%5D2.0.CO;2
- Cokendolpher JC, Krejca JK (2010) A new cavernicolous *Paribisium* Chamberlin 1930 (Pseudoscorpiones: Neobisiidae) from Yosemite National Park, U.S.A., Museum of Texas Tech University 297: 1–25. https://doi.org/10.5962/bhl.title.156953
- Culver DC, Master LL, Christman MC, Hobbs HH III (2000) Obligate cave fauna of the 48 contiguous United States. Conservation Biology 14: 386–401. https://doi.org/10.1046/j.1523-1739.2000.99026.x

- Ćurčić BPM (1983) A revision of some Asian species of *Microcreagris* Balzan, 1892 (Neobisiidae, Pseudoscorpiones). Bulletin of the British arachnological Society 6 (1): 23–36.
- Deuve T, Tian MY (2018) Nouveaux Trechini cavernicoles du Guizhou ord-occidental des genres Zhijinaphaenops et Guizhaphaenops (Coleoptera, Trechidae). Bulletin de la Société entomologique de France 123 (3): 333–339. https://doi.org/10.32475/bsef_2039
- Feng ZG, Wynne JJ, Zhang F (2019) Two new subterranean-adapted pseudoscorpions (Pseudoscorpiones: Neobisiidae: *Parobisium*) from Beijing, China. Zootaxa 4661 (1): 145–160. https://doi.org/10.11646/zootaxa.4661.1.7
- Ferreira RL, Horta LCS (2001) Natural and human impacts on invertebrate communities in Brazilian caves. Revista Brasileira de Biologia 61: 7–17. https://doi.org/10.1590/S0034-71082001000100003
- Fišer C, Zagmajster M, Zakšek V (2013) Coevolution of life history traits and morphology in female subterranean amphipods. Oikos 122: 770–778. https://doi.org/10.1111/j.1600-0706.2012.20644.x
- Gao ZZ, Chen HM, Zhang F (2017) Description of two new cave-dwelling *Bisetocreagris* species (Pseudoscorpiones: Neobisiidae) from China. Turkish Journal of Zoology 41: 615–623. https://doi.org/10.3906/zoo-1602-39
- Gao ZZ, Wynne JJ, Zhang F (2018) Two new species of cave-adapted pseudoscorpions (Pseudoscorpiones: Neobisiidae, Chthoniidae) from Guangxi, China. Journal of Arachnology 46: 345–354. https://doi.org/10.1636/JoA-S-17-063.1
- Guo XB, Zhang F (2016) Two new species of the genus *Parobisium* Chamberlin, 1930 from China (Pseudoscorpiones: Neobisiidae). Entomologica Fennica 27: 140–148.
- Hanski I, Ovaskainen O (2002) Extinction debt at extinction threshold. Conservation Biology 16: 666–673. https://doi.org/10.1046/j.1523-1739.2002.00342.x
- Harley GL, Polk JS, North LA, Reeder PP (2011) Application of a cave inventory system to stimulate development of management strategies: The case of west-central Florida, USA. Journal of Environmental Management 92: 2547–2557. https://doi.org/10.1016/j.jenvman.2011.05.020
- Harvey MS (1991) Catalogue of the Pseudoscorpionida. Manchester University Press: Manchester, Pp. 850.
- Harvey MS (1992) The phylogeny and classification of the Pseudoscorpionida (Chelicerata: Arachnida). Invertebrate Taxonomy 6: 1373–1435. https://doi.org/10.1071/ IT9921373
- He W, Li P (2016) Resources features of Guizhou Karst cave and development and utilization. Journal of Guizhou Normal University (Natural Sciences) 2016, 34 (03): 1–6 (in Chinese with English abstract). https://doi.org/10.16614/j.cnki.issn1004-5570.2016.03.001
- Hoff CC (1961) Pseudoscorpions from Colorado. Bulletin of the American Museum of Natural History 122: 409–464.
- Hong Y (1996) Two new species of the genus *Parobisium* (Pseudoscorpionida: Neobisiidae) from Korea. Korean Journal of Systematic Zoology 12: 189–197.
- Huang SB, Cen YJ, Tian MY (2017) A new genus and a new subgenus of cavernicolous beetles from Furong Jiang valley, southwestern China (Coleoptera: Carabidae: Trechinae). Annales de la Société entomologique de France (N.S.) 53 (4): 286–295. https://doi.org/10.1080/0 0379271.2017.1344566

- Howarth FG (1980) The zoogeography of specialized cave animals: A bioclimatic model. Evolution 34: 394–406. https://doi.org/10.1111/j.1558-5646.1980.tb04827.x
- Howarth FG (1983) Ecology of cave arthropods. Annual Review of Entomology 28: 365–389. https://doi.org/10.1146/annurev.en.28.010183.002053
- Howarth FG, James SA, McDowell W, Preston DJ, Imada CT (2007) Identification of roots in lava tube caves using molecular techniques: Implications for conservation of cave arthropod faunas. Journal of Insect Conservation 11: 251–261. https://doi.org/10.1007/ s10841-006-9040-y
- Judson MLI (2007) A new and endangered species of the pseudoscorpion genus Lagynochthonius from a cave in Vietnam, with notes on chelal morphology and the composition of the Tyrannochthoniini (Arachnida, Chelonethi, Chthoniidae). Zootaxa 1627: 53–68. https:// doi.org/10.11646/zootaxa.1627.1.4
- Kováč ĽU, Mock AN, Ľuptáčik PE, Košel VL, Fenďa PE, Svatoň J, Mašán P (2005) Terrestrial arthropods of the Domica Cave system and the Ardovská Cave (Slovak Karst)–principal microhabitats and diversity. 7th Central European Workshop on Soil Zoology, April 14-16, 2003, In *Contributions to Soil Zoology in Central Europe I*, České Budějovice, 61–70.
- Li B, Zhao Z, Zhang C, Li S (2019a) *Troglocoelotes* gen. n., a new genus of Coelotinae spiders (Araneae, Agelenidae) from caves in South China. Zootaxa 4554 (1): 219–238. https://doi.org/10.11646/zootaxa.4554.1.7
- Li SQ, Wang XX (2017) New cave-dwelling spiders of the family Dictynidae (Arachnida: Araneae) from Guangxi and Guizhou, China. Zoological Systematics 42 (2): 125–228
- Li YC, Shi AM, Liu H (2017) A new cave-dwelling species of *Bisetocreagris* (Arachnida, Pseudoscorpiones: Neobisiidae) from Yunnan Province, China. Entomol. Fennica 28: 212– 218. https://doi.org/10.33338/ef.84688
- Li YC, Liu H, Shi AM (2019b) A new cave-dwelling species of *Lagynochthonius* (Arachnida: Pseudoscorpiones: Chthoniidae) from Yunnan Province, China. Zootaxa 4571 (1): 28–34. https://doi.org/10.11646/zootaxa.4571.1.2
- Mahnert V (2003) Four new species of pseudoscorpions (Arachnida, Pseudoscorpiones: Neobisiidae, Chernetidae) from caves in Yunnan Province, China. Revue Suisse de Zoologie 110: 739–748. https://doi.org/10.5962/bhl.part.80209
- Mahnert V (2009) New species of pseudoscorpions (Arachnida, Pseudoscorpiones, Chthoniidae, Chernetidae) from caves in China. Revue Suisse de Zoologie 116: 185–201. https:// doi.org/10.5962/bhl.part.79492
- Mahnert V, Li YC (2016) Cave-inhabiting Neobisiidae (Arachnida: Pseudoscorpiones) from China, with description of four new species of *Bisetocreagris* Ćurčić. Revue Suisse de Zoologie 123: 259–268.
- Mammola S, Cardoso P, Culver DC, Deharveng L, Ferreira RL, Fišer C, Galassi DPM, Griebler C, Halse S, Humphreys WF, Isaia M, Malard F, Martinez A, Moldovan OT, Niemiller ML, Pavlek M, Reboleira ASPS, Souza-Silva M, Teeling EC, Wynne JJ, Zagmajster M (2019) Scientists' warning on the conservation of subterranean ecosystems. BioScience 69 (8): 641–650. https://doi:10.1093/biosci/biz064.
- Mammola S, Goodacre SL, Isaia M (2018) Climate change may drive cave spiders to extinction. Ecography 41: 233–243.https://doi.org/10.1111/ecog.02902

- Morikawa K (1960) Systematic studies of Japanese pseudoscorpions. Memoirs of Ehime University (2B) 4: 85–172.
- Price L (2016) An introduction to some cave fauna of Malaysia and Thailand. Acta Carsologica 33: 311–317. https://doi.org/10.3986/ac.v33i1.359
- Ran JC, Yang WC (2015) A review of progress in Chinese troglofauna research. Journal of Resources and Ecology 6: 237–246. https://doi.org/10.5814/j.issn.1674-764x.2015.04.007
- Rong L, Yang L (2004) Biodiversity of Guizhou Province and its karst environment. Journal of Guizhou Normal University (Natural Sciences) 2004, (04): 1–6 (in Chinese with English abstract). https://doi.org/10.3969/j.issn.1004-5570.2004.04.001
- Schawaller W (1995) Review of the pseudoscorpion fauna of China (Arachnida: Pseudoscorpionida). Revue Suisse de Zoologie 102: 1045–1063. https://doi.org/10.5962/bhl.part.80489
- Sket B (2008) Can we agree on an ecological classification of subterranean animals? Journal of Natural History 42: 1549–1563. https://doi.org/10.1080/00222930801995762
- Song Y, Zhao HF, Luo YF, Li SQ (2017) Three new species of *Pinelema* from caves in Guangxi, China (Araneae, Telemidae). ZooKeys 692: 83–101. https://doi.org/10.3897/zookeys.692.11677
- Stone FD, Howarth FG (2007) Hawaiian cave biology: status of conservation and management. Proceedings of the 2005 National Cave and Karst Management Symposium; Albany, New York, October 31 - November 4, 2005. Pp. 21–26.
- Taylor SJ, Krejca J, Smith JE, Block VR, Hutto F (2003) Investigation of the potential for red imported fire ant (*Solenopsis invicta*) impacts on rare karst invertebrates at Fort Hood, Texas: A field study. In Illinois Bexar County Karst Invertebrates Draft Recovery Plan Natural History Survey. Center for Biodiversity Technical Report, vol. 28. Pp. 1–153.
- Tian MY, Huang SB, Wang DM (2017) Discovery of a most remarkable cave-specialized trechine beetle from southern China (Coleoptera, Carabidae, Trechinae). ZooKeys 725: 37–47. https://doi.org/10.3897/zookeys.725.21040
- Tian MY, Huang SB, Wang DM (2018) Occurrence of hypogean trechine beetles in Hanzhong Tiankeng Cluster, southwestern Shaanxi, China (Coleoptera: Carabidae: Trechinae). Annales de la Société entomologique de France (N.S.) 54 (1): 81–87. https://doi.org/10.108 0/00379271.2017.1417056
- Tian MY, Huang SB, Wang XH, Tang MR (2016) Contributions to the knowledge of subterranean trechine beetles in southern China's karsts: five new genera (Insecta: Coleoptera: Carabidae: Trechini). ZooKeys 564: 121–156. https://doi.org/10.3897/zookeys.564.6819
- Tilman D, May RM, Lehman CL, Nowak MA (1994) Habitat destruction and the extinction debt. Nature 371: 65–66. https://doi.org/10.1038/371065a0
- Trajano E (2000) Cave faunas in the Atlantic tropical rain forest: Composition, ecology and conservation. Biotropica 32: 882–893. https://doi.org/10.1111/j.1744-7429.2000. tb00626.x
- van Beynen P, Townsend K (2005) A disturbance index for karst environments. Environmental Management 36: 101–116. https://doi.org/10.1007/s00267-004-0265-9
- Vellend M, Verheyen K, Jacquemyn H, Kolb A, Van Calster H, Peterken G, Hermy M (2006) Extinction debt of forest plants persists for more than a century following habitat fragmentation. Ecology 87: 542–548. https://doi.org/10.1890/05-1182

- Voituron Y, de Frapoint M, Issartel J, Guillaume O, Clobert J (2010) Extreme lifespan of the human fish (*Proteus anguinus*): A challenge for ageing mechanisms. Biology Letters 7: 105–107. https://doi.org/10.1098/rsbl.2010.0539
- Whitten T (2009) Applying ecology for cave management in China and neighboring countries. Journal of Applied Ecology 46: 520–523. https://doi.org/10.1111/j.1365-2664.2009.01630.x
- Wynne JJ, Howarth FG, Sommer S, Dickson BG (2019) Fifty years of cave arthropod sampling: techniques and best practices. International Journal of Speleology 48: 33–48. https://doi.org/10.5038/1827-806X.48.1.2231
- Zhou ZF, Zhang SY, Xiong KN, Li B, Tian ZH, Chen Q, Yan L, Xiao SZ (2017) The spatial distribution and factors affecting karst cave development in Guizhou Province. Journal of Geographical Sciences 27 (8): 1011–1024. https://doi.org/10.1007/s11442-017-1418-0

Subterranean Biology 34: 99–108 (2020) doi: 10.3897/subtbiol.34.50916 http://subtbiol.pensoft.net

SHORT COMMUNICATION



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

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

l 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)

Academic editor: O. T. Moldovan | Received 7 February 2020 | Accepted 18 March 2020 | Published 15 May 2020

http://zoobank.org/5F5FA394-FFAD-403F-A741-E967722A1294

Citation: Rassam H, Moutaouakil S, Benaissa H, Albrecht C, Ghamizi M (2020) First record of *Pisidium subtruncatum* Malm, 1855 (Bivalvia, Sphaeriidae) in an African cave. Subterranean Biology 34: 99–108. https://doi.org/10.3897/ subtbiol.34.50916

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 μ m 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)



Figure I. 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).

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

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* occurence.



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.

Ta	bl	е	2.	Measurements	of	internal	s	hell	features.
----	----	---	----	--------------	----	----------	---	------	-----------

	Ν	L	Н	LA	LP	LE	LL	LH	HH
$Mean \pm SD$	4	2.96 ± 0.81	2.28 ± 0.53	1.8 ± 0.64	1.16 ± 0.31	0.81 ± 0.24	0.48 ± 0.09	1.44 ± 0.38	0.15 ± 0.05

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

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



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 (C_2) and the uppermost (C_4) parallelly located, C_4 overlaps C_2 at anterior end, C_3 is long and slightly curved (Fig. 5). All individuals found in the present work are exactly similar



Figure 5. Position and shapes of cardinal teeth and ligament pits in left (a) and right valve (b). c: cardinal teeth.

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 6. Map of occurrence localities of *Pisidium subtruncatum* from this paper and previous record (red triangles).

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 (Bespalaya 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 lightsensitive 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

- Adam W (1960) Faune de Belgique: Mollusques Terrestres et Dulcicoles. Institut Royal des Sciences Naturelles de Belgique, Bruxelles, 402 pp.
- Alhejoj A, Bandel K, Salameh EM (2017) Aquatic Mollusks: Occurrences, Identification and Their Use as Bioindicators of Environmental Conditions (Salinity, Trace Elements and Pollution Parameters) in Jordan. Water Ressources in Arid Areas: The Way Forward. Springer Water, 295–318. https://doi.org/10.1007/978-3-319-51856-5_17
- Anderson RV (1977) Concentration of cadmium, copper, lead, and zinc in six species of freshwater clams. Bulletin of Environmental Contamination and Toxicology 18: 492–496. https://doi.org/10.1007/BF01683722
- Bespalaya Y (2015) Molluscan fauna of an Arctic lake is dominated by a cosmopolitan *Pisidium* species. Journal of Molluscan Studies 81: 294–298. https://doi.org/10.1093/mollus/eyu081

- Boessneck U, Clewing C, Albrecht C (2016) Exploring high-mountain limnic faunas: discovery of a novel endemic bivalve species (Sphaeriidae: *Pisidium*) in the Nepal Himalayas. Invertebrate Systematics 30: 588–597. https://doi.org/10.1071/IS15043
- Cofrancesco AF (2002) Nervous System and Sense Organs in Bivalves. Zebra Mussel Research Program.
- Colgan DJ, Ponder WF, Eggler PE (2000) Gastropod evolutionary rates and phylogenetic relationships. Zoologica Scripta 29: 29–63. https://doi.org/10.1046/j.1463-6409.2000.00021.x
- Culver DC (2012) Mollusks Encyclopedia of Caves (2nd edn.). American University, 512–517. https://doi.org/10.1016/B978-0-12-383832-2.00074-8
- Doat J, Boutonnet S, Rozier F, Boutonnet S, Molinaro M, Mouysset B, Dalmayrac S, Dohin D, Salmon R, Mago C, Nespoulous C (2005) Expédition de la Fédération Française de Spéléologie. Comité Départemental de Spéléologie du Tarn, Fédération Française de Spéléologie.
- Gadzała-Kopciuch R, Berecka B, Bartoszewicz J, Buszewski B (2004) Some considerations about bioindicators in environmental monitoring. Polish Journal of Environmental Studies 13: 453–462.
- García N, Cuttelod A, Abdul Malak D (2010) The Status and Distribution of Freshwater Biodiversity in Northern Africa. IUCN, Gland, Cambridge, Malaga, 141 pp. https://doi. org/10.2305/IUCN.CH.2009.19.en
- Groh K, Bößneck U, Clewing C, Albrecht C, Richling I (2020) Discovery of a new pill clam from an unusual habitat – the interstitial *Pisidium interstitialis* n. sp. from Southwestern and Central Germany: (Bivalvia: Sphaeriidae). Journal of Molluscan Studies 86: 1–16. https://doi.org/10.1093/mollus/eyz036
- Horsák M (2001) Současný stav našich hrachovek (*Pisidium*) a možnosti jejich využití v bioindikaci [The present status of our pill clams and possibilities of their application in bioindication]. Ochrana přírody 56: 53–56.
- Ingram WM, Ballinger DG, Gauffin AR (1953) Relationship of Sphaerium solidulum Prime to organic pollution. Ohio Journal of Science 53: 320–235. https://doi. org/10.1111/j.1949-8594.1953.tb07008.x
- Killeen I, Aldridge D, Oliver G (2004) Freshwater Bivalves of Britain and Ireland. Field Studies Council (1st edn.), 119 pp.
- Knight LRFD (2018) The aquatic invertebrate fauna of selected Scottish caves. The Grampian Speleological Group Bulletin 2: 19–37.
- Knight LRFD, Wood PJ (2000) Bivalves (Pisidiidae) in English caves. Cave and Karst Sciences 27: 89–90.
- Korniushin AV (2000) Review of the family Sphaeriidae (Mollusca: Bivalvia) of Australia, with the description of four new species. Records of Australian Museum 52: 41–102. https:// doi.org/10.3853/j.0067-1975.52.2000.1308
- Kuiper JGJ (1972) Une récolte de *Pisidium* dans le Moyen Atlas. Résultats de la mission biologique au Maroc de l'Université de Gand, Belgique. Basteria 36: 2–5.
- Kuiper JGJ (1983) The Sphaeriidae of Australia. Basteria 47: 3-52.
- Medcof JC, Kerswill CJ (1965) Effects of Light on Growth of Oysters, Mussels, and Quahaugs. Journal of the Fisheries Research Board of Canada 22: 281–288. https://doi.org/10.1139/ f65-030

- ONSSA (2018) Code de Procédure, Direction de Contrôle des Produits Alimentaires: Conditions d'utilisation des eaux. ONSSA, Rabat, 14 pp.
- Piechocki A (1989) The Sphaeriidae of Poland (Bivalvia, Eulamellibranchia). Annales Zoologici 24: 249–320.
- Prié V (2019) Molluscs. In: White W, Culver D, Pipan T (Eds) Encyclopedia of Caves. 725– 731. https://doi.org/10.1016/B978-0-12-814124-3.00087-X
- Rohlf FJ (2003) tpsRelw, relative warps analysis, version 1.36. Department of Ecology and Evolution, State University of New York at Stony Brook. https://life.bio.sunysb.edu/ee/rohlf/software.html
- Rohlf FJ (2005) tpsDig, digitize landmarks and outlines, version 2.05. Department of Ecology and Evolution, State University of New York at Stony Brook. https://life.bio.sunysb.edu/ ee/rohlf/software.html
- Vargovitsh RS, Anistratenko VV (2016) "*Pisidium zoctanum* Poli, 1876" (Mollusca, Bivalvia) a ghost-taxon from the Crimean Karani-Koba Cave. Ruthenica 26: 171–174.
- Vinarski MV, Kantor YI (2016) Analytical catalogue of fresh and brackish water molluscs of Russia and adjacent countries. KMK Scientific Press, Moscow, 544 pp.
- Wilke T, Davis GM, Qiu D, Spear RC (2006) Extreme mitochondrial sequence diversity in the intermediate schistosomiasis host *Oncomelania hupensis robertsoni*: another case of ancestral polymorphism? Malacologia 48: 143–157.
- Wurtz CB (1955) Stream biota and stream pollution. Sewage and Industrial Wastes 27: 1270–1278.
- Zhang Z, Schwartz S, Wagner L, Miller W (2000) A greedy algorithm for aligning DNA sequences. Journal of Computational Biology 7: 203–214. https://doi. org/10.1089/10665270050081478
RESEARCH ARTICLE



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

José G. Palacios-Vargas¹

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)

Academic editor: O. T. Moldovan R	eceived 31 January 2020	Accepted 29 April 2020	Published 27 May 2020
http://zooban	k.org/52274932-8D7D-4DA	C-94D8-9DF52EB60ACA	

Citation: Palacios-Vargas JG (2020) *Acheroxenylla* (Collembola, Hypogastruridae), first record from the Americas with description of a new species from a Peruvian cave. Subterranean Biology 34: 109–119. https://doi.org/10.3897/subtbiol.34.50673

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 macrosetae, 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). Postantennal 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 + 4or 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
_	Furcula and retinaculum well developed or reduced, but always present3
2	2 + 2 eyes, occasionally with small pigment spots; p3 on Abd. IV present
_	2 + 2 eyes, always with blue pigment spots; p3 on Abd. IV ab-
	sent
	lands: 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.

112

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 welldeveloped 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 μ m with small barbulations. Sensorial setae longer than regular setae, about 30 μ m. 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.



Figures 1–4. I *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.

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 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



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



Figure 10. Acheroxenylla lipsae sp. nov. Dorsal abdominal chaetotaxy.

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



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.

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*.

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 tenent hair (A1), sometimes weakly clavate, is similar to the new species. The ungues 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 ungues.

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 *Acheroxenylla*, and also specimens of *Folsomina* sp., *Pseudosinella* sp., *Trogolaphysa* sp., *Cyphoderus* sp., *Isotomurus* sp., *Pararrhopalites* palites 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 - troglofile. *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.

Acknowledgments

The molecular part of this contribution was done thanks to the project "Application of metabarcoding tools to long-term monitoring of soil fauna" (Yves Basset & Héctor Barrios), funded by SENACYT FID16-070. Dr. Diana Silva Dávila, Dept. Entomology; Museo de Historia Natural, Universidad Mayor de San Marcos, Perú kindly lend the cave material collected by Josiane Lips for study. Yony Callohuari (Museo de Entomología Klaus Raven Büller – Universidad Nacional Agraria La Molina) made the slides. Blanca Mejía mounted the cuticles recovered from the sequence's extraction. Jair Paéz made some drawings under the microscope and prepared the final drawing plates (Fac. Ciencias, UNAM). The Franco-Peruvian Scientific Expedition "Cerro Blanco 2017" lead this study, Dr Jean-Loup Guyot, Dr Xavier Robert, of the Research Institute for Development (IRD), and James Apaéstegui, of the Instituto Geofísico from Perú (IGP). This speleological expedition was organized and supported by Spéléologique Groupe of Bagnols-Marcoule (Gard, France), the Vulcain Caving Club (Lyon, France) and the Espeleo Club Andino (Lima, Peru), the IRD and the IGP. Dr. Frans Janssens reviewed the manuscript and gave important comments to improve it. Larry La Rose (Tres Cruces, N. M. USA) reviewed the final manuscript.

References

- Bellinger PF, Christiansen KA, Janssens F (1996–2019) Checklist of the Collembola of the World. http://www.collembola.org
- Bocanegra Buitrón TR (2014) Lista Taxonómica comentada de los Collembola de Perú con aportes a su distribución geográfica. Universidad Nacional Federico Villarreal, Perú. Facultad de Ciencias Naturales y Matemática. Escuela profesional de Biología. Tesis de Licenciatura, 78 pp.
- Ellis WN (1976) Autumn fauna of Collembola from Central Crete. Tijdschrift voor Entomologie, 119(8): 221–326.
- Fjellberg A (1992) Collembola of the Canary Islands. 1. Introduction and survey of the family Hypogastruridae. Entomologica Scandinavica 22(4): 437–456. https://doi. org/10.1163/187631291X00246
- Najt J, Thibaud J-M (1987) Collemboles (Insecta) de l'Equteur. 1. Hypogastruridae, Neanuridae et Isotomidae. Bulletin du Museum National d'histore Naturelle, Section A Zoologie Biologie et Ecologie Animales 9(1): 201–209.
- Thibaud J-M, Schulz H-J, da Gama Assalino MM (2004) Synopses on Palaearctic Collembola, Volume 4. Hypogastruridae. Abhandlungen und Berichte des Naturkundemuseums Görlitz 75(2): 1–287.
- Ratnasingham S, Hebert PD (2013) A DNA-based registry for all animal species: the Barcode Index Number (BIN) system. PloS ONE 8(8): e66213. https://doi.org/10.1371/journal. pone.0066213