European Journal of Taxonomy 891: 87–109
https://doi.org/10.5852/ejt.2023.891.2273
ISSN 2118-9773
www.europeanjournaloftaxonomy.eu
2023 · Camacho A.I. *et al.*

Research article

urn:lsid:zoobank.org:pub:BAB17D92-2A57-45FF-91D5-E3A3F3F6F10F

Description of a new species of *Hobbsinella* (Crustacea, Bathynellacea, Bathynellidae) from Colorado (USA) based on morphological and molecular characters

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Abstract. A new species of Bathynellidae is described from Colorado (USA). *Hobbsinella gunnisonensis* Camacho & Taylor sp. nov. displays a unique combination of morphological characters including seven-segmented antenna lacking medial seta on exopod, antennule slightly longer than antenna, three-segmented mandibular palp, four articles on endopod of thoracopods I to VII and five spines on sympod and three spines on endopod of the uropods. Partial sequences of cytochrome oxidase I (COI) and 18S have been obtained from several specimens of the new species. The mitocondrial and nuclear DNA data complement

the traditional morphological taxonomic description support the validity of the new species. Molecular data for the Bathynellidae demonstrate the presence of two highly divergent genetic units, with the new species placed in the genus *Hobbsinella*. With the description of *Hobbsinella gunnisonensis* Camacho & Taylor sp. nov. and its molecular characterization, we discovered an interesting distribution of the genus, which occurs in both sides of the Continental Divide (Texas and Colorado) and different habitats.

Keywords. Bathynellacea, Bathynellidae, Colorado, groundwater fauna, new species, morphological data, COI, 18S.

Camacho A.I., Mas-Peinado P., Bonwell C.B., McDonald J.N., Dorda B.A., Rey I. & Taylor S.J. 2023. Description of a new species of *Hobbsinella* (Crustacea, Bathynellacea, Bathynellidae) from Colorado (USA) based on morphological and molecular characters. *European Journal of Taxonomy* 891: 87–109.
https://doi.org/10.5852/ejt.2023.891.2273

Introduction

The crustacean family Bathynellidae Grobben, 1905 is widespread throughout the world, including 36 genera and 109 species (Camacho *et al*. 2021). This family has been poorly studied in North America (Camacho *et al*. 2018a), only nine species from three genera are known (Camacho *et al*. 2018b): three species of the genus *Bathynella* Vejdovsky, 1882 (from California and Colorado), five species of the genus *Pacificabathynella* Schminke & Noodt, 1988 (from California, Montana and Alaska) and one species of the genus *Hobbsinella* Camacho *et al.*, 2018, from Texas. Bathynellidae show great morphological homogeneity and convergence due to the environmental pressures of the habitat where they live (groundwater). Therefore, character identification to differentiate species and genera is quite challenging. Molecular techniques can be particularly useful in providing additional information to help delimit within Bathynellidae.

1. The genus *Bathynella* isn't properly described.
2. Many species have not been adequately described and need to be revisited and therefore comparisons are very difficult.
3. *Bathynella* became, with time, the "catch-all" genus where most of the described Bathynellidae have been placed.
4. So far, *B. riparia* Pennak & Ward, 1985 is the only species described for Colorado but needs revision.

In this paper, we describe a new species of the genus *Hobbsinella* originally from Texas. To support the morphological descriptions, we obtained sequences of mitochondrial (COI) and nuclear (18S) DNA from several specimens. With the description of *Hobbsinella gunnisonensis* Camacho & Taylor sp. nov. and its molecular characterization, the genus range is broadened in North America. *Hobbsinella gunnisonensis* sp. nov. is found in the West of the Continental Divide and *H. edwardensis* Camacho *et al.*, 2018, is found East of the Continental Divide in a different habitat type and at a markedly lower elevation. We expand the distribution species of this genus, now known to span a range of more than 1300 km between type localities, from Texas to Colorado.

Material and methods

Study area and groundwater sampling method

The study area covers the whole world because the genera studied come from different parts of Europe, North America, Asia and Australia. Species of Bathynellidae are distributed mostly in groundwater of caves from Spain (*Vejdovskybathynella* Serban & Leclerc, 1984 and *Paradoxiclamousella* Camacho, Dorda & Rey, 2013), France (*Gallobathynella* Serban, Coineau & Delamare Deboutteville, 1971) and UK (*Antrobathynella* Serban, 1966); artesian wells from Texas (*Hobbsinella* Camacho *et al.* 2018); springs from Slovenia (*Bathynella* sp.); mixing and hyporheic zones of surface rivers from Italy (*B. ruffoi*

Serban, 1973, *B*. cf. *ruffoi*), Colorado (*Hobbsinella gunnisonensis* sp. nov., *Bathynella riparia* Pennak & Ward, 1985) and Russia (*Altainella* Camacho, 2020) and bore holes from Australia (*Pilbaranella* Perina & Camacho, 2018, *Fortescuenella* Perina & Camacho, 2019, *Anguillanella* Perina & Camacho, 2019 and *Muccanella* Perina & Camacho, 2019) (see Table 1). Bathynellids have been collected by the authors and other colleagues (A. Brancelj, L. Knight, G. Perina, C. Bou, S. Iepure, B. Newell and B. Hutchins) using various sampling methods (Camacho 1992): Bou-Rouch tube, Karaman-Chappuis, net hand, phreatobiological net (Cvetkov 1968), and baited bottle traps (Perina *et al*. 2018, 2019a, 2019b; Camacho 2019).

In the summer of 2018, field surveys of mixing zone and hyporheic waters were sampled with a Bou Rouch pump across the Colorado Rockies (Fig. 1A). Sites (n = 50) were stratified within USEPA HUC8 basins in the Southern Rockies Level IV Ecoregion (Fig. 1A), with specific locations determined by accessibility and feasibility of sampling. The Watershed Boundary Dataset from the U.S. Geological Survey – National Geospatial Program (2021) was used to delineate drainage basins. The map (Fig. 1) was generated in QGIS (ver. 3.20.1-Odense, 2021), with further processing in Affinity Designer (ver. 2.0.4, 2023). At each field site, three to four replicate samples were collected at haphazardly selected locations, at least 2 m apart and in substrate suitable for the sampling apparatus. For each sample, we used a Bou-Rouch GW pump, collecting 10 l of water which was filtered through a fine mesh aquarium net. Samples were preserved in 95% ethanol and kept in a cooler until transported to the laboratory for storage in a freezer. Samples were sorted in the laboratory (Bonwell *et al*. 2019) and bathynellaceans, found at only 2 of the 50 sites, were shipped from Colorado to the senior author of this paper (AIC) for morphological and molecular anaylyses. The 11 specimens of the new species described here were collected from the mixing and/or hyporheic zone of the Lottis Creek (eight specimens) and Spring Creek (three specimens) (Gunnison County), both sites are tributaries of the River Gunnison (Fig. 1A) (Table 2)

The type locality is Lottis Creek off Forest Road 742, Gunnison County, Colorado.

The fauna found together with the bathynellaceans in samples included riffle beetles (Coleopetera: Elmidae), biting midges (Diptera: Ceratopogonidae), midges (Diptera: Chironomidae), crane flies (Diptera: Tipulidae), mayflies (Ephemeroptera), stoneflies (Plecoptera), caddisflies (Trichoptera), water mites (Trombidiformes: Hydrachnidia) and aquitic oligochaetes (Tubificida: Naididae).

Specimens used for morphological study were later fixed in 4% buffered formalin and stored in 70% ETOH. Specimens used for molecular study were directly frozen at -20°C, in 400 ml of distilled water or 0.5 ml digestion buffer. Five DNA extracts (one whole specimens and four abdomens, corresponding to four specimens of the type series and one of the additional material) of the new species are used in the molecular analysis (Tables 1–2).

Morphological study

Morphological descriptions are based on the holotype (female), type series (6 specimens) and additional material (three specimens).

For the morphological and molecular study of the new species, *Hobbsinella gunnisonensis* sp. nov., eleven females were used (see Tables 1–2 for specimen details, vouchers and GenBank numbers). The abdomen of six specimens were removed and used to extract DNA together with one whole specimen (Table 2). Eight specimens constitute the morphological type series of the new species described herein. A complete dissection of all appendages was performed and the resultant body parts were preserved as permanent slides (special metal slides, glycerine-gelatine stained with methylene blue and paraffin as mounting medium; see Perina & Camacho 2016). The morphological examination was performed using an oil immersion lens (at 1000× magnification) with a Zeiss interference microscope. Drawings were done using a drawing tube, digitalised using a WACOM Tablet and retouched using Freehand and/or Adobe Illustrator drawing software.

Table 1 (continued on next page). Specimens used in the molecular analyses (*type locality). Abbreviations: asl = above sea level; E = East; m = meters; MNCN = Museo Nacional de Ciencias Naturales de Madrid (Spain); MNCN/ADN = Tissues and DNA collection of the MNCN; N = North; WAMC = Western Australian Museum Collection (in bold); Z = altitude.

Species	Locality	Coordinates			Voucher	GenBank accession number	
		N	E	Z	MNCN/ADN WAMC	18S	COI
Iberobathynella imuniensis Camacho, 1987 OUTGROUP	*Torca Morteros Cave, Burgos, Spain	43.14786	-3.59539	1285	29166	KC469528	HQ659850
1. *Altainella calcarata* Camacho, 2020	Aktru River, Altai Mountain, Russia	50.06944	87.75028	2408	54879	MN262081	MN258523
2. *Anguillanella callawaensis* Perina & Camacho, 2019	CA0006, bore hole, Western Australia	-20.64445	120.30626	–	**57258**	MF042209	MF074337
3. *Antrobathynella stammeri* (Jakobi, 1954)	Ogof Cave, UK	51.831159	-3.686986	–	54711	MF094714	MF114307
4. *Bathynella* cf *ruffoi*	Dobbiaco Lake, Italy	46.70682	12.21926	1260	54719	MF436212	MF443327
5. *Bathynella* cf *ruffoi*	Aurino River, Gais, Italy	46.84611	11.95019	850	54727	MF436213	MF443328
6. *Bathynella ruffoi* Serban, 1973	*Rienza River, San Sigismondo, Italy	46.81054	11.80044	770	54729	MF436214	MF443329
7. *Bathynella* sp.	Kamniška Cave, Slovenia	46.32747	14.59031	592	54668	MF094716	MF114309
8. *Bathynella* sp.	Trubarjeva Cave, Slovenia	46.36142	13.77056	1854	54718	MF094715	MF114308
9. *Fortescuenella serenitatis* Perina & Camacho, 2019	* SOM049, bore hole, Western Australia	-22.14167	117.5294	–	**57315**	MK134939	MK134992
10. *Gallobathynella boui* Serban, Coineau & Delamare Deboutteville, 1971	*La Devèze Cave, Courniou, France	43.47455	2.71417	365	54600	KP999757	KP974146
11. *Hobbsinella edwardensis* Camacho & Hutchins 2018	Edwards Aquifer, Hays County, Texas, USA	29.88958	-97.93645	177	29943	KP999685	–
12. *Hobbsinella edwardensis*	Edwards Aquifer, Hays County, Texas, USA	29.88958	-97.93645	177	54640	–	MF443323
13. *Hobbsinella gunnisonensis* sp. nov.	*Spring Creek, Gunnison County, Colorado, USA	38.75418	-106.76984	2622	54883	MN262078	MN258520
14. *Hobbsinella gunnisonensis* sp. nov.	*Lottis Creek, Gunnison County, Colorado, USA	38.77145	-106.62195	2797	54886	MN262077	MN258519
15. *Hobbsinella gunnisonensis* sp. nov.	*Lottis Creek, Gunnison County, Colorado, USA	38.77145	-106.62195	2797	54887	OQ812159	OQ940715
16. *Hobbsinella gunnisonensis* sp. nov.	*Lottis Creek, Gunnison County, Colorado, USA	38.77145	-106.62195	2797	54888	OQ812160	OQ940716
17. *Hobbsinella gunnisonensis* sp. nov.	*Lottis Creek, Gunnison County, Colorado, USA	38.77145	-106.62195	2797	54889	OQ812161	OQ940717
18. *Muccanella cundalinensis* Perina & Camacho, 2019	CU0046, bore hole, Western Australia	-20.54346	120.15983	–	**57340**	MN149125	MN136082

Table 1 (continued). Specimens used in the molecular analyses (*type locality). Abbreviations: asl = above sea level; E = East; m = meters; MNCN = Museo Nacional de Ciencias Naturales de Madrid (Spain); MNCN/ADN = Tissues and DNA collection of the MNCN; N = North; WAMC = Western Australian Museum Collection (in bold); Z = altitude.

Species	Locality	Coordinates			Voucher	GenBank accession number	
		N	E	Z	MNCN/ADN WAMC	18S	COI
19. *Paradoxiclamousella fideli* Camacho, Dorda & Rey, 2013	CO220, Carnero Spring Cave, Cantabria, Spain	43.28680	-4.47784	156	29735	KC469523	JX121252
20. *Paradoxiclamousella fideli*	*CO069, Pozo Agua Cave, Asturias, Spain	43.28383	-4.52318	374	29746	KC469524	JX121253
21. *Paradoxiclamousella fideli*	CO209, Treslajorá Cave, Cantabria, Spain	43.26725	-4.59085	1132	29594	JX121235	JX121249
22. *Pilbaranella ethelensis* Perina & Camacho, 2018	* W088 Pilbara, bore hole, Western Australia	-23.39647	119.82126	–	**57292**	MF042239	MF074338
23. *Vejdovskybathynella caroloi* Camacho, 2007	Gándara Cave, Cantabria, Spain	43.19096	-3.58635	760	29877	KC469525	KC469538
24. *Vejdovskybathynella edelweiss* Camacho, 2007	*Ojo Guareña Cave (OG01), Burgos, Spain	43.03188	-3.65821	724	29478	KP999677	HQ596565
25. *Vejdovskybathynella edelweiss*	Ojo Guareña Cave (OG16), Burgos, Spain	43.03188	-3.65821	724	29414	KP999676	HQ596570
26. *Vejdovskybathynella edelweiss*	Huesos Cave, Cornejo, Burgos, Spain	43.03231	-3.63881	705	29440	KC469513	HQ596571
27. *Vejdovskybathynella vasconica* Camacho, Dorda & Rey, 2013	*Goikoetxe Cave, Vizcaya, Spain	43.36026	-2.70195	149	29646	KC469521	KC469535
28. *Vejdovskybathynella* sp. 1	Erizos River, Ojo Guareña Cave, Burgos, Spain	43.03188	-3.65821	724	29487	KC469514	HQ596572
29. *Vejdovskybathynella* sp. 1	Río Chico Cave, Cantabria, Spain	43.19088	-3.58071	573	29728	KC469522	JX121244
30. *Vejdovskybathynella* sp. 1	Sogalamuela Cave, Burgos, Spain	43.03236	-3.62914	668	103487	OQ812158	OQ940714
31. *Vejdovskybathynella* sp. 2	Redonda Cave, Burgos, Spain	43.03236	-3.62914	668	29523	KC469515	HQ596573

The material is deposited in the Arthropod collection of MNCN (ARTP/MNCN).

Repositories

CSIC	=	Consejo Superior de Investigaciones Científicas, Madrid, Spain
MNCN	=	Museo Nacional de Ciencias Naturales de Madrid, Spain
MNCN/ADN	=	Tissues and DNA collection of the MNCN
MNCN/ARTP	=	Arthropods collection of the MNCN
WAMC	=	Western Australian Museum Collection, Perth, WA, Australia

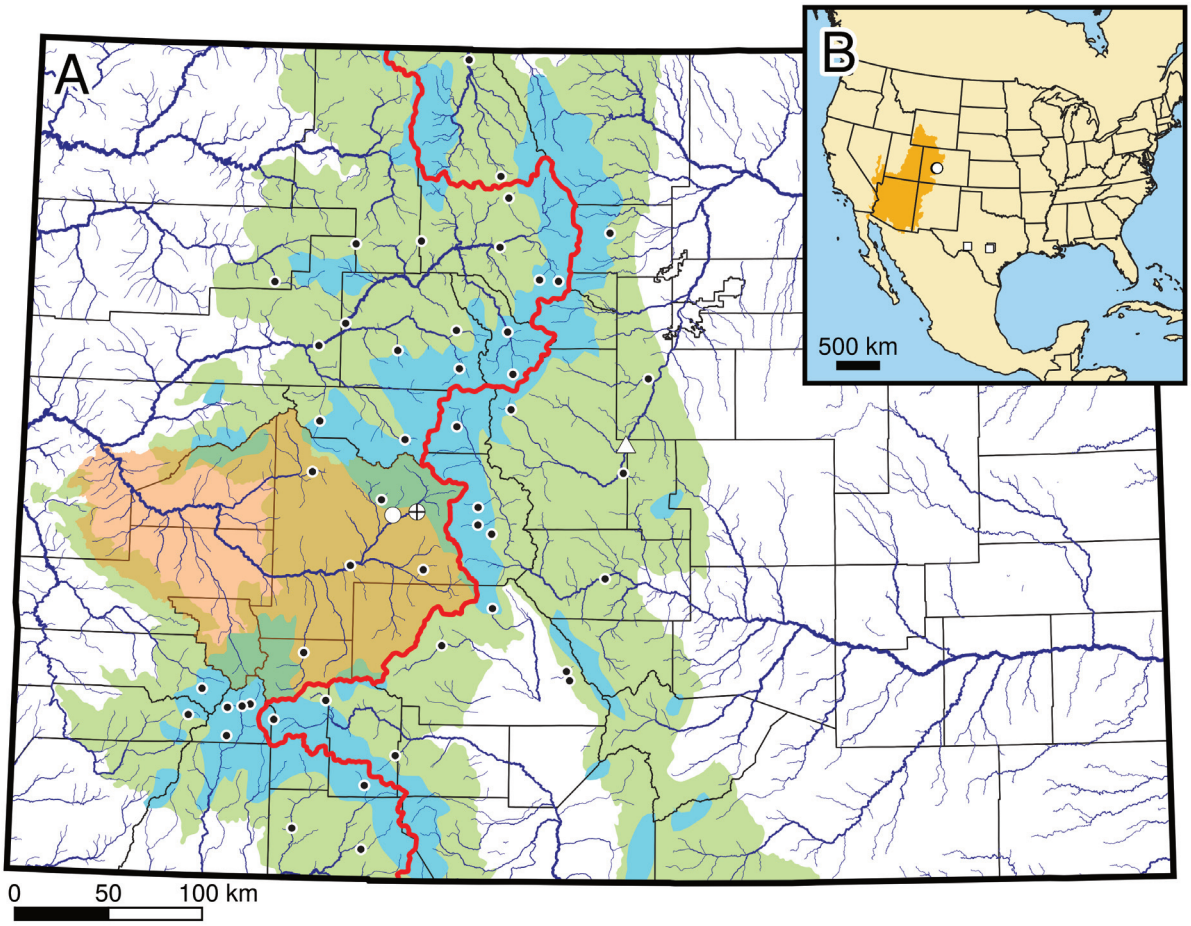

Fig. 1. Field study area, showing the type locality (Lottis Creek, cross in circle). **A**. State of Colorado with known bathynellaceans *Hobbsinella gunnisonensis* Camacho & Taylor sp. nov. (white circles, cross in circle for type locality) and *Bathynella riparia* Pennak & Ward, 1985 (white triangle). Small black circles: 48 streambed sites sampled during the summer 2018 using a Bou-Rouch pump where Bathynellacea Chappuis, 1915 were not detected. Light blue shaded areas: approximate Late Pleistocene glacial extent from Leonard (2007: fig. 2). Pink-brown shading: the Gunnison River Drainage Basin. Green-Southern Rockies US EPA Level 3 Ecoregion. Stream order (3–7) indicated by dark blue line width, 1st and 2nd order streams not shown. Red line: continental divide. Colorado counties shown as thin black lines. **B**. Conterminous United States in North America, with distributions of *Hobbsinella edwardensis* Camacho *et al.*, 2018 (squares) and *Hobbsinella gunnisonensis* (circles). Colorado River Drainage Basin indicated in darker brown.

Table 2. Specimens of *Hobbsinella gunnisonensis* Camacho & Taylor sp. nov. studied from Gunnison County (Colorado). Collectors: C.N. Bonwell & J.J. McDonald. Voucher number of MNCN Artropods (MNCN/ARTP) and Tissues and DNA (MNCN/ADN) Collections. Abbreviation: No = sequencing failed.

	Locality and sampling site	Sample and lot (sex specimens)	Voucher MNCN/ARTP 20.04	Size mm	Voucher MNCN/ADN	18S	COI
Holotype	Lottis Creek; CO-137	CO-137-4; CO-137-4-5 (♀)	20172	1.12	–	–	–
Paratype	Lottis Creek; CO-137	CO-137-4; CO-137-4-5 (♀)	20173	1.03	–	–	–
Paratype	Lottis Creek; CO-137	CO-137-4; CO-137-4-5 (♀)	20174	1.21	–	–	–
Paratype	Lottis Creek; CO-137	CO-137-2; CO-137-2-4 (♀)	20178	1.00	54885 (abdomen)	No	No
Paratype	Lottis Creek; CO-137	CO-137-3; CO-137-3-6 (♀)	20179	1.00	54886 (abdomen)	Yes	Yes
Paratype	Lottis Creek; CO-137	CO-137-4; CO-137-4-5 (♀)	20180	1.00	54887 (abdomen)	Yes	Yes
Paratype	Lottis Creek; CO-137	CO-137-4; CO-137-4-5 (♀)	20181	1.00	54888 (abdomen)	Yes	Yes
Paratype	Lottis Creek; CO-137	CO-137-4; CO-137-4-5 (♀)	–	–	54889 (whole)	Yes	Yes
Type series	Spring Creek; CO-136	CO-136-3; CO-136-3-10 (♀)	20175	1.00	54883 (abdomen)	Yes	Yes
Type series	Spring Creek; CO-136	CO-136-3; CO-136-3-10 (♀)	20176	1.00	54884 (abdomen)	No	No
Type series	Spring Creek; CO-136	CO-136-3; CO-136-3-10 (♀)	20177	1.00	–	–	–

The morphological terminology used throughout the text follows Serban (Serban 1972; Schminke & Noodt 1988).

Abbreviations used in text and tables

A = absent
AI = antennule
AII = antenna
Art = article
E = East
Endp = endopod
Exp = exopod
Ha = habitat
L = large
M = medium size
Md = mandible
Mx.I = maxillule
Mx.II = maxilla
N = North
P = present
S = small
Symp = sympod
Th I–VIII = thoracopods I-VIII
TL = type locality
Urp = uropod
XL = extra large
Z = altitude

Molecular analysis

DNA extraction, amplification, and sequencing

DNA extraction and amplification methods have been described in Camacho *et al.* (2020). The samples were placed in 0.5 ml digestion buffer (Gilbert *et al*. 2007), and incubated overnight at 55°C with gentle agitation. Buffer consisted of 5 mM $CaCl_2$, 2% sodium dodecyl sulphate (SDS), 40 mM dithiotreitol (DTT), 250 mg/ml proteinase K, 10 mM Tris buffer pH 8, 2.5 mM EDTA (Ethylene-Diamine-Tetra-Acetic acid) pH 8.0, and 10 mM NaCl (final concentrations). After incubation, nucleic acids were extracted from the digestion buffer using a Qiaquick PCR purification kit (QIAGEN) (Alda *et al*. 2007).

Partial sequence of the COI gene were amplified with the primers C1-J-1718 and HCO2198 (Folmer *et al*. 1994; Simon *et al*. 1994) for all specimens. 18S rRNA partial sequences were amplified in three fragments, using the primers 1F and 3R; 3F and 5R and 5F and 9R (Giribet *et al*. 1996). We used 3 µl of DNA extract for template. Other components of the 25 µl PCR reaction included 1 × of the corresponding buffer (75 mM Tris HCl, pH 9.0; 50 mM KCl and 20 mM $(NH_4)_2SO_4$), 2 mM $MgCl_2$, 10 mM dNTPs mix, 0.1 µM of both primers, 0.02% BSA, and 0.125 units AmpliTaq Gold® DNA Polymerase (Applied Biosystems). The PCR program consisted of an initial denaturation step of 95°C for 10 min, followed by 60 amplification cycles (95°C for 30 s, 45–49°C for 45 s and 72°C for 45 s) and a final elongation step of 72°C for 10 min. PCR were run on an Eppendorf Mastercycler gradient. PCR product (5 µl) was electrophoresed through a 1.5% agarose gel and visualized with SYBR SafeTM DNA Gel Stain (Invitrogen) under ultraviolet light. PCR products were purified by treatment with ExoSAP-IT (USB Amersham, Buckinghamshire, UK) and incubated at 37°C for 45 min, followed by 80°C for 15 min to inactivate the enzyme. Purified PCR products were sequenced in both directions using the BigDye Terminator ver. 3.1 sequencing kit (Applied Biosystems Inc., Foster City, USA) in a 10 µl volume, containing 15–20 ng purified product and 3 pmol primer (Camacho *et al*. 2016).

DNA extractions have been deposited in the Tissues and DNA Collection of the MNCN. Voucher numbers of the seven specimens of the new species used in the molecular analyses are shown in Table 2.

Phylogenetic analysis

To explore the phylogenetic relationships within Bathynellidae, we use partial sequences of the mtDNA gene cytochrome oxidase 1 (COI) (509 bp) and the nuclear 18S rRNA (1071 bp) from a total of 31 specimens. These data sets include 11 clearly identified genera (*Vejdovskybathynella*, *Paradoxiclamousella*, *Gallobathynella*, *Bathynella*, *Hobbsinella*, *Antrobathynella*, *Altainella*, *Pilbaranella*, *Fortescuenella*, *Anguillanella* and *Muccanella*) and 18 well identified species (Table 1). We used *Iberobathynella imuniensis* Camacho, 1987 as outgroup representative of the Bathynellidae's sister lineage of Parabathynellidae Noodt, 1965 to root the phylogenetic analysis. All sequences used were submitted to GenBank (see Table 1 for locality, collection voucher number and GenBank Accession Number for each specimen).

All sequences were compiled and edited using Geneious ver. 10.2.4 (https://www.geneious.com) (Kearse *et al*. 2012). Sequences were aligned using the MAFFT (Multiple Alignment using Fast Fourier Transform) algorithm (Katoh & Toh 2008), as implemented in Geneious and the final alignment was checked with Geneious and Mesquite ver. 3.04 (Maddison & Maddison 2015) for gaps and translate it to see if stop codons were present. Sequence divergences (uncorrected p-distances) of 18S, among genera, and COI, among species, were calculated using PAUP* ver. 4.0b 10 (Swofford 2002).

Sequences obtained were then compared with sequences from GenBank (mostly generated by the authors of this paper over many years of wok) using Blast (Altschul *et al.* 1997).

Phylogenetic analyses were carried out using Maximum Likelihood (ML) and Bayesian Inference (BI) approaches, analyzing both the COI and 18S datasets separately and together. The ML analyses were conducted in IQ-TREE ver. 2.1.1 (Minh *et al*. 2020). We estimated the best partition scheme with the option MFP-MERGE in ModelFinder (Kalyaanamoorthy *et al.* 2017), with the following parameters: low perturbation strength (-pers 0.2), number of unsuccessful iterations to stop (-nstop) set to 500 and, to assess node support, 2000 ultrafast bootstrap replicates. The BI analyses were run in MsBayes ver. 3.2.6 (Ronquist *et al.* 2012) as implemented in CIPRES Science Gateway ver. 3 (Miller *et al*. 2010). The substitution model space was explored with the reversible-jump model (option lset nst = mixed rates = invgamma; Huelsenbeck *et al*. 2004). Two independent analyses were run with one cold and three heated chains, each chain ran for 100 million generations, with the first 25% discarded as burnin. From the resulting trees, a 50% majority rule consensus tree was obtained. The consensus phylogenetic tree was then edited in FigTree ver. 1.4.3. (http://tree.bio.ed.ac.uk/software/figtree).

Results

Systematic account

Class Malacostraca Latreille, 1802
Order Bathynellacea Chappuis, 1915
Family Bathynellidae Grobben, 1904
Subfamily Gallobathynellinae Serban, Coineau & Delamare Deboutteville, 1971

Genus ***Hobbsinella*** Camacho, Hutchins, Schwartz, Dorda, Casado & Rey, 2018

Type species

Hobbsinella edwardensis Camacho, Hutchins, Schwartz, Dorda, Casado & Rey, 2018

Amended diagnosis

AI and AII seven-segmented. AII longer than AI. Md palp sexually-dimorphic. Endopod of ThI to VII each four-segmented. Male ThVIII of globular aspect; penial region with two small lobes, inner lobe, and a frontal projection, and with a group of small denticles at base; basipod large, vertical, with frontal crest provided with teeth and lateral rim of spines and one distal seta and a large outer protuberance completly integrated on coxopod; exopod very elongated, as long as preceding thoracopods, with five setae; endopod with two setae of different size. Female ThVIII with a reduced 'seta' on coxopod or without seta; epipod very large; exopod longer than endopod. Uropod: sympod with five spines; endopod with three or four spines. Furcal rami with second spine sligthly longer than rest, which are all equal in length.

Hobbsinella gunnisonensis Camacho & Taylor sp. nov.
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Figs 2–4

Etymology

The species name, *gunnisonensis* (adjective, patronym), is derived from the Gunnison River Drainage Basin in the headwaters of the Colorado River where the new species occurs in two tributaries of the Gunnison River.

Material examined

Holotype

USA • ♀; Colorado, Gunnison County, Lottis Creek off Forest Road 742; 38.77145° N, 106.62195° W; 3 Jul. 2018; C.B. Bonwell and J.N. McDonald leg.; dissection of all appendages and body parts preserved as permanent slides (special metal slides), glycerine-gelatine stained with methylene blue and paraffin as mounting medium; sample CO-137-4, CO-137-4-5; MNCN/ARTP20.04/20172.

Paratypes

USA • 5 ♀♀; same collection data as for holotype; dissection of all appendages and body parts preserved as permanent slides (special metal slides), glycerine-gelatine stained with methylene blue and paraffin as mounting medium; sample CO-137-4, CO-137-4-5; MNCN/ARTP20.04/20173, MNCN/ARTP20.04/20174, MNCN/ARTP20.04/20180 (MNCN/ADN54887), MNCN/ARTP20.04/20181 (MNCN/ADN54888), MNCN/ADN54889 • 1 ♀; same collection data as for holotype; dissection of all appendages and body parts preserved as permanent slides (special metal slides), glycerine-gelatine stained with methylene blue and paraffin as mounting medium; sample CO-137-3, CO-137-3-6; MNCN/ARTP20.04/20179 (MNCN/ADN54886) • 1 ♀; same collection data as for holotype; dissection of all appendages and body parts preserved as permanent slides (special metal slides), glycerine-gelatine stained with methylene blue and paraffin as mounting medium; sample CO-137-2, CO-137-2-4; MNCN/ARTP20.04/20178 (MNCN/ADN54885).

Other material examined

USA • 3 ♀♀; Colorado, Gunnison County, Spring Creek off Forest Road 744; 38.75418° N, 106.76984° W; 3 Jul. 2018; C.B. Bonwell and J.N. McDonald leg.; dissection of all appendages and body parts preserved as permanent slides (special metal slides), glycerine-gelatine stained with methylene blue and paraffin as mounting medium; sample CO-136-3, CO-136-3-10; MNCN/ARTP20.04/20175 (MNCN/ADN54883), MNCN/ARTP20.04/20176 (MNCN/ADN54884), MNCN/ARTP20.04/20177.

Description

Measurements and appearance. Body total length of holotype 1.12 mm. Total length of females 1.0–1.21 mm. Body elongated, articles widening slightly towards posterior end, approximately ten times as long as wide. Head longer than wide. Pleotelson with one small barbed dorsal seta on each side.

Antennules (AI) (Fig. 2A). Seven-segmented; first three articles almost as long as last four articles combined; first article bit longer than the last article, which is more slender than the other articles; fourth article very short, fifth and sixth equal in length; inner flagellum almost square; setation as in Fig. 2A article six with three aesthetascs, similar in size; seventh article with three aesthetascs similar in size. AI slightly shorter than AII.

Antennae (AII) (Fig. 2B). Seven-segmented; first article similar in length to the fourth; second and third articles are the shortest, while the fifth is slightly longer and just over the half length of the distal article setal formula: 0+0/1+0/2+1/2+0/0+0/2+2/5.

Labrum (Fig. 2C). Almost trapezoidal; with smooth free edge and a median cleft.

Paragnaths (Fig. 2D). Almost rectangular, globose, with a very strong claw on distal part; dense setulation on distal half.

Mandibles (Md) (Fig. 2E–F). Palp with three articles, third article (Fig. 2E) with two strong barbed claws, first and third article almost square, second article elongated. Masticatory part (Fig. 2E): incisor process (pars incisiva) with two teeth; processus incisivus accessorius with one tooth and one small seta-like tooth; pars molaris with one tooth, nearest to processus incisivus accessorius, bidentate, and with two dentate structures, parallel to main axis of teeth, each with two small denticles and with a strong distal tooth.

Maxillules (MxI) (Fig. 2G). Proximal endite with four setae, three of them setulose; distal endite with six teeth (four with denticles and two seta-like); three plumose setae of different lenght, one longer than the other two, in outer margin.

Maxillae (MxII) (Fig. 2H). Four articles; setal formula 8, 5, 6, 6.

Thoracopods (ThI to VII) (Figs 3A–E, 4A–B). Well developed ThI to III (Fig. 3A-C) progressively longer; ThIV (Fig. 3D) and V (Fig. 3E) of similar length; ThVI and VII (Fig. 4A–B) similar and a little longer than rest. Th I without epipod, coxa with long strong plumose seta, basipod with three smooth setae and tuft of long fine setules near base. Epipod present on ThII-VII, more than half length of basipod in all Ths. Exopod with one article in all Ths, shorter than endopod, similar in length to first three endopodal articles combined in ThI–V, slightly longer than first two articles combined in ThVI and VII, with five barbed setae (two terminal, one dorsal and two ventral). Endopod four-segmented in all thoracopods, all articles large and subequal in length in ThI to V; endopod of ThIII to V similar and slightly longer than in ThI–II and V, second and third articles very long in ThVI and VII; setal formula of endopods (number of setae on basipod in brackets):

ThI: (3) 4+0/3+1/2+0/5
ThII–III: (2) 2+0/2+1/2+0/4
ThIV–V: (1) 1+0/1+1/1+0/4
ThVI–VII: (1) 0+0/0+1/0+0/2(1)

Thoracopod VIII (ThVIII) (Fig. 4C). Coxa without small seta; large and thick epipod, as long as basipod; endopod one-segmented with two unequal apical setae one smooth and one plumose; exopod two times as long as endopod, with three smooth similar setae, one of these subterminal and two other terminals.

Pleopods (Fig. 4D). Two segmented; first article with very long plumose seta; second article with five setae of different length.

Pleotelson (Fig. 4E). With one long, plumose dorsal seta at each side near base of furca.

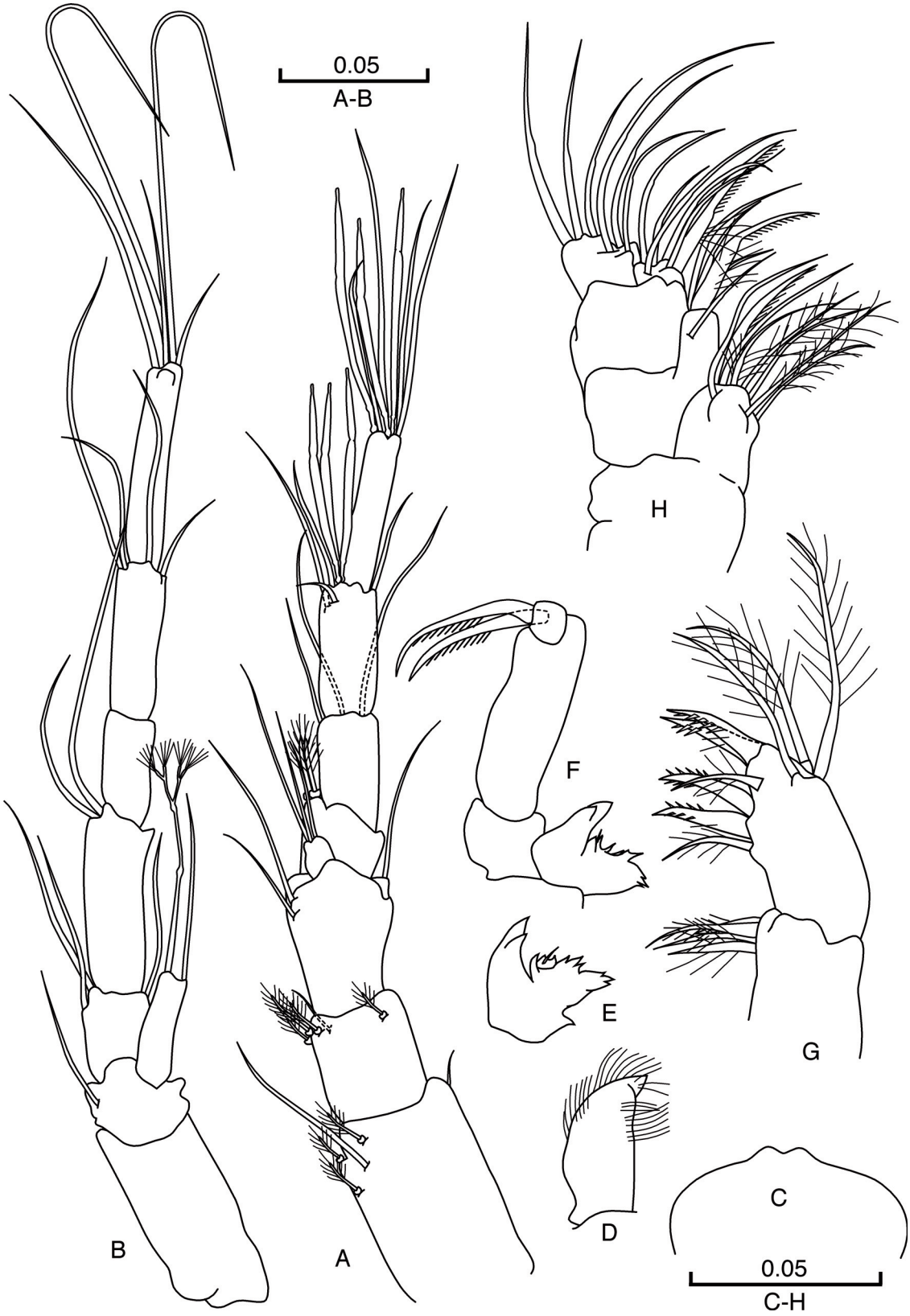

Fig. 2. *Hobbsinella gunnisonensis* Camacho & Taylor sp. nov., holotype, ♀ (MNCN/ARTP20.04/20172). **A**. AI. **B**. AII. **C**. Labrum. **D**. Paragnath. **E**. Masticatory part of Md. **F**. Md. **G**. MxI. **H**. MxII. Scale bar in mm.

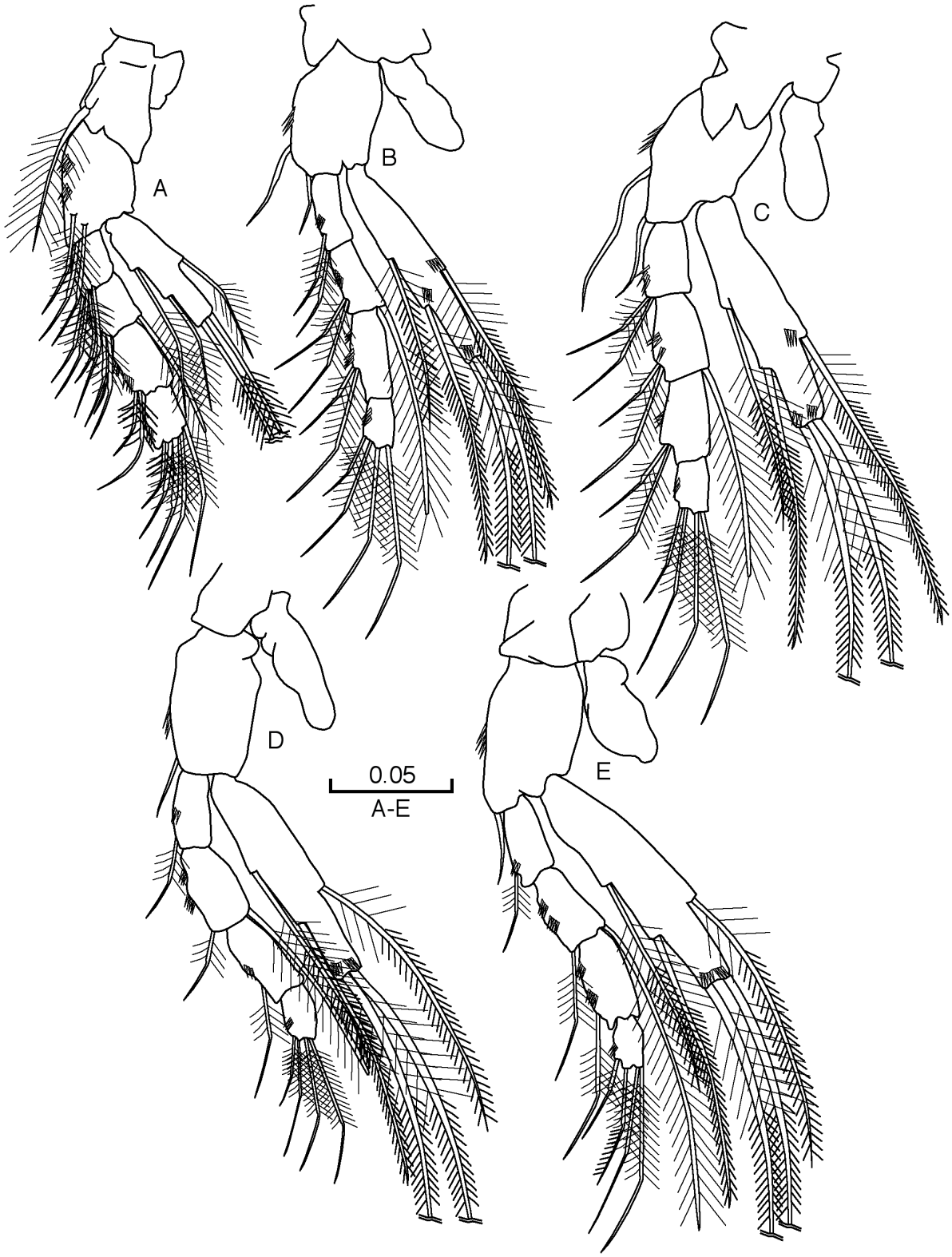

Fig. 3. *Hobbsinella gunnisonensis* Camacho & Taylor sp. nov., holotype, ♀ (MNCN/ARTP20.04/20172). **A**. ThI. **B**. ThII. **C**. ThIII. **D**. ThIV. **E**. ThV. Scale bar in mm.

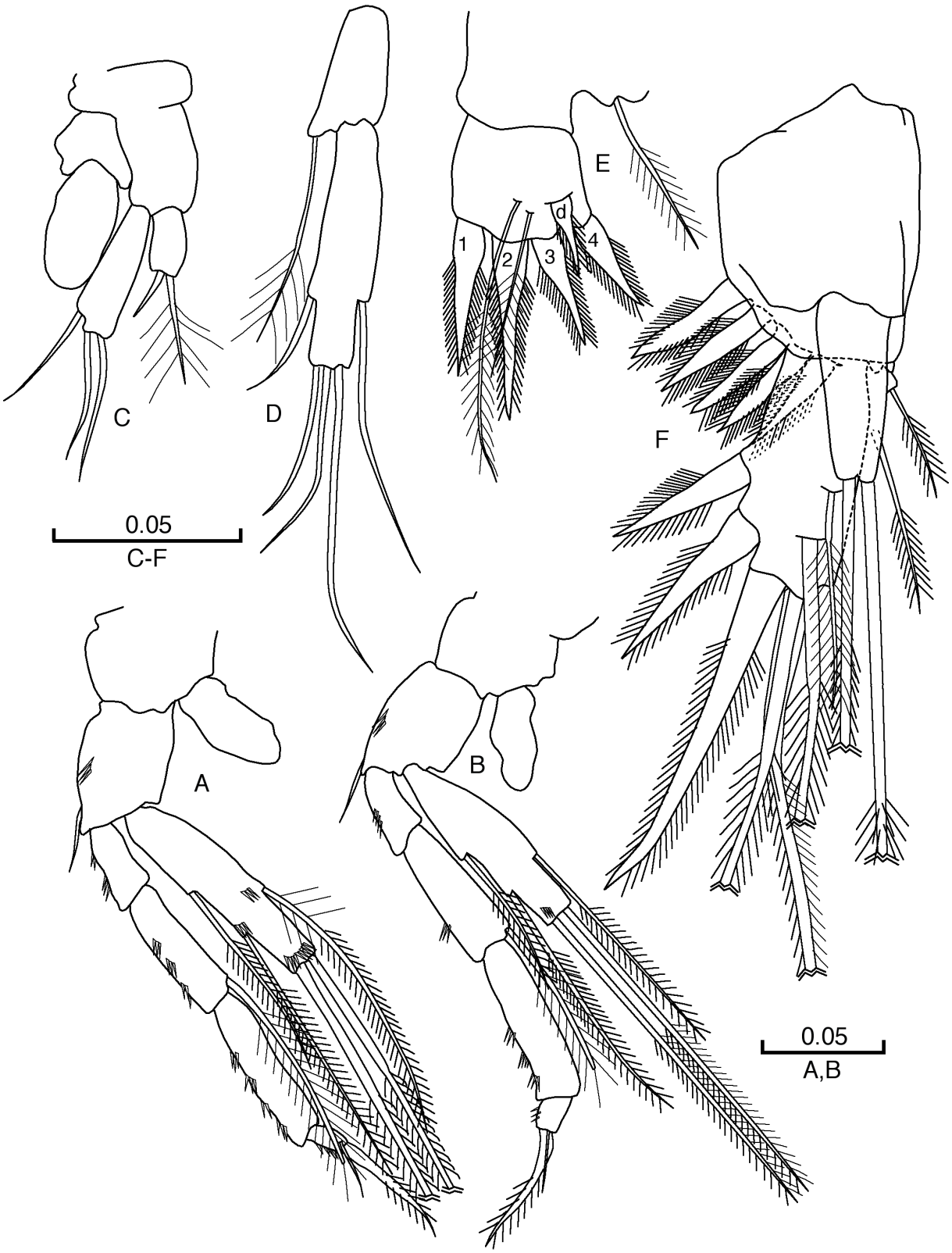

Fig. 4. *Hobbsinella gunnisonensis* Camacho & Taylor sp. nov., holotype, ♀ (MNCN/ARTP20.04/20172). **A**. ThVI. **B**. ThVII. **C**. ThVIII. **D**. Pleopod. **E**. Furcal ramus, dorsal view. **F**. Uropod, dorsal view. Scale bar in mm.

Furcal rami (Fig. 4E). Almost square, bearing five short spines of similar size except second, which is slightly longer than rest and the dorsal, which is slightly shorter.

Uropods (Fig. 4F). Sympod, almost square, as long as endopod, with five long, equal distal spines; endopod almost 20% longer than exopod, with three strong claws (basal two subequal in length, distal claw twice as long as the others), three very long distal barbed setae (Fig. 4I) and one plumose setae located dorsolaterally; exopod with four setae (two terminal and two medial).

Remarks

The new species shares the combination of morphological characters listed in the diagnosis with the type species of the genus *Hobbsinella*, described originally from Texas (Fig. 1B): seven-segmented AI and AII; pars molaris of mandible with two parts; endopod of all thoracopods four-segmented; female thoracopod VIII biramous and a very large epipod. The genus presents some peculiarities with respect to other North American and European genera, as already highlighted when describing the genus from Texas (Camacho *et al*. 2018b). The new species maintains those peculiarities of the type species but it is worth clarifying some of them. For example the fourth and last articles of AII in the species of *Hobbsinella* are longer than in other species of the American and European genera. The arrangement of the teeth on the pars molaris of the mandible is also unique. The female ThVIII has a very long epipod that exceeds the length of the basipod, but it is not bulky as in other genera with long epipods. The pleopod consists of two articles, as in all species of the family, but in most species, the first article is generally short (less than half the length of the second). In *Hobbsinella*, the first article of the pleopod is two thirds the length of the second. The spines of the sympod of the uropod are fairly long and not very thick, unlike most genera with shorter, thicker sympodal spines. The morphological differences between the species are difficult to find. The new species is slightly smaller than the type species, *H. edwardensis*. *Hobbsinella gunnisonensis* sp. nov. AII is slightly longer than AI (as *Vandelibathynella* Serban, Coineau & Delamare Deboutteville, 1971) while in *H. edwardensis* the great length of the AII is very striking, which far exceeds the length of AI (Table 3) (Camacho *et al*. 2018b). The third article of AI has only three setae on the new species, five in *H. edwardensis*, and also three aesthetacs on article six (only two in the type species), but the setal formula of the rest of articles is similar in both species and both species lacks of medial seta on exopod of AII. Both species differs also in the combinations of setae on the articles of the endopod of ThI to V, as well as the combinations of setae on the basipod of all thoracopods (Table 3). The differences between the two species are subtle and very difficult to appreciate, since in general they refer to the size, appearance and relative proportions of the different articles. The number of spines and/or setae on MxII, Ths and uropods differ between the two species, and these differences are summarized (Table 3) to facilitate comparison.

Molecular results

18S rRNA and COI sequences were obtained from five females specimens of the new species (Table 2).

The concatenated COI–18S data set is represented by 31 sequences of 1580 bp. 509 bp COI and 1071 bp 18S sequences were obtained from 31 specimens.

The uncorrected sequence divergence estimates for 18S between genera and the outgroup within the family Bathynellidae are consistent with previous studies (Camacho *et al*. 2016, 2018a, 2018b, 2020, 2021; Perina *et al.* 2019a, 2019b). For 18S, sequence divergence between the genera of Bathynellidae and the outgroup used in the phylogeny (*Iberobathynella imuniensis* Parabathynellidae family) ranged between 7.4–8.2% (*Gallobathynella*), 8.0% (*Altainella*) and 12.3% (*Pilbaranella*). *Hobbsinella* shows a 18S divergence range with *Gallobathynella* and *Vejdovskybathynella* of 5.4%–5.7% and 5–6%–7.8% with *Paradoxiclamousella* and 9.0%–15.4% with Australian genera (Perina *et al*. 2019a).

Table 3. Differences amongst the two species known of the genus *Hobbsinella* Camacho *et al*., 2018. Abbreviations: A = absent; AI = antennule; AII = antenna; art = article; endp = endopod; exp = exopod; P = present.

	H. edwardensis	***H. gunnisonensis*** sp. nov.
AI: aesthetacs on articles 6/7	2/3	3/3
setae on article 3	5	3
AI/AII	AI<<AII	AI<AII
AII: setal formula	0+0/1+0/2+0/2+0/0+0/2+2/5	0+0/1+0/2+1/2+0/0+0/2+2/5
Md: first and third articles of palp	rectangular	almost square
MxI: setules on outer margin	P	A
MxII: setal formula	7/4/7/5	8/5/6/6
ThI: (setae basipod) setae art endp	(4) 4+0/2+1/2+0/4	(3) 4+0/3+1/2+0/5
ThII: (setae basipod) setae art endp	(2) 3+0/2+1/2+0/4	(2) 2+0/2+1/2+0/4
ThIII: (setae basipod) setae art endp	(2) 2+0/2+1/2+0/4	(2) 2+0/2+1/2+0/4
ThIV: (setae basipod) setae art endp	(1) 2+0/2+1/2+0/4	(1) 1+0/1+1/1+0/4
ThV: (setae basipod) setae art endp	(1) 2+0/2+1/1+0/4	(1) 1+0/1+1/1+0/4
ThVI–VII: basipod	rectangular, large	almost square
(setae basipod) setae art endp	(1) 0+0/0+1/0+0/2(1)	(1) 0+0/0+1/0+0/2(1)
Female ThVIII: coxal seta	P	A
Epipod	two times basipod	as basipod
Exopod	almost 3 times endopod	2 times endopod
Setae	2	3
Pleopod: setae	1+6	1+5
Uropod: sympod	rectangular	almost square
sympod	25% longer than endp	as long as endp
endp	40% longer than exp	20% longer than exp
claws	4	3
setae	5	4
exop: setae	5	4
Furca: ratio second/first spines	all similar	1.5 times longer
Dorsal spines	= all	dorsal = the smallest
Dorsal seta of pleotelson	as long as furca	longer than furca
Maximum female length	1.6	1.21

The uncorrected sequence divergence estimates for COI between the new species and *H. edwardensis* is 8.5–8.7% and is consistent with the values found between congeneric species such as *Vejdovskybathynella edelweiss* Camacho, 2007 (different populations), *V. vasconica* Camacho, Dorda & Rey, 2013 (14.7%) and *V. caroloi* Camacho, 2007 that shows a COI divergence ranged between 6.5–7.5% or between *B. ruffoi* Serban, 1973 and other European *Bathynella* undetermined (4.6–6.2%). Between populations of the new species the difference found is 0.3%, less than between populations of *V. edelweiss* (0.5–0.6%).

Maximum Likelihood (ML) (bootstrap support, BS) and Bayesian Inference (BI) (posterior probabilities, PP) phylogenetic analyses recovered similar topologies (Fig. 5), supporting two monophyletic clades corresponding to genera of Bathynellidae from Australia and the other genera (PP = 0.94; BS = 79). European and North American genera form two well supported, monophyletic lineages (PP = 1; BS = 100). One of these correspond to the subfamily Bathynellinae Grobben, 1905 and the other to the subfamily Gallobathynellinae Serban, Coineau & Delamare Deboutteville, 1971 in which the new species is placed. The subfamily Gallobathynellinae comprises species of the genera *Vejdovskybathynella* (PP = 0.9, BS = 82) and *Paradoxiclamousella* (PP = 1, BS = 100) from the Iberian Peninsula, *Gallobathynella* from France and *Hobbsinella* (PP = 1, BS = 100) from the USA with two well differentiated and supported clades (PP = 1, BS = 100). One clade includes *H. edwardensis* from Texas and the other includes the specimens of *H. gunnisonensis* sp. nov. from the two localities in Colorado. The subfamily Bathynellinae includes species of *Bathynella* from Slovenia and Italy; *Antrobathynella stammeri* (Jakobi, 1954) from the UK and *Altainella calcarata* Camacho *et al.*, 2020 from Russia.

The four species described from Western Australia (*Anguillanella callawaensis* Perina & Camacho, 2019, *Fortescuenella serenitatis* Perina & Camacho, 2019a, *Muccanella cundelinensis* Perina & Camacho, 2019 and *Pilbaranella ethelensis* Perina & Camacho, 2018) could represent Austrobathynellinae Delamare Deboutteville & Serban, 1973, but molecular data of the original species for which the subfamily was created are needed to confirm this.

Molecular data support morphology and the decisión to create a new species

Discussion and conclusion

In groups such as bathyllenaceans, where morphological simplification is extreme, the validity of new species based exclusively on morphology may be questionable, specially in the absence of specimens of both sexes to complete comparisons. In this paper, we present all the morphological characters needed to establish the new species, although based only on females, because the molecular information obtained supports the decision to create a new species, *H. gunnisonensis* sp. nov., genetically distant from *H. edwardensis*. The molecular divergence (COI uncorrected p-distance) between the two species of *Hobbsinella* is 8.5–8.7%, which is lower than COI p-distance found amongst some European species of the genus *Vejdovskybathynella* (between 12–14%) (Camacho *et al*. 2011), but is higher than, for example, that found between species of the genus *Brevisomabathynella* Cho, Park & Ranga Reddy, 2006 (about 6%) (Abrams *et al*. 2012) of the family Parabathynellidae, therefore molecular data support the morphology and the decisión to erect a new species from only females. The use of COI thresholds is helpful in identifying potential new species, but their description should be based on multiple lines of evidence integrating molecular and morphological data.

The molecular data provide the phylogenetic position of *H. gunnisonensis* sp. nov. as a sister species of *H. edwardensis*. The genus *Hobbsinella* is confirmed as sister group of the European genera of the subfamily Gallobathynellinae, well differentiated from the subfamily Bathynellinae, the other clade which includes *Bathynella*, *Antrobathynella* and *Altainella*. There are three major monophyletic and well-supported groups: Gallobathynellinae and Bathynellinae subfamilies, and the Australian bathynellids.

The basal Australian clade is formed by four genera, *Pilbaranella*, *Muccanella*, *Fortescuenella* and *Anguillanella* and corroborates previous results (Camacho *et al.* 2018a, 2018b, 2020, 2021; Perina *et al*. 2019a, 2019b). The taxonomic position of the new species seems clear based on our detailed morphological and molecular analysis.

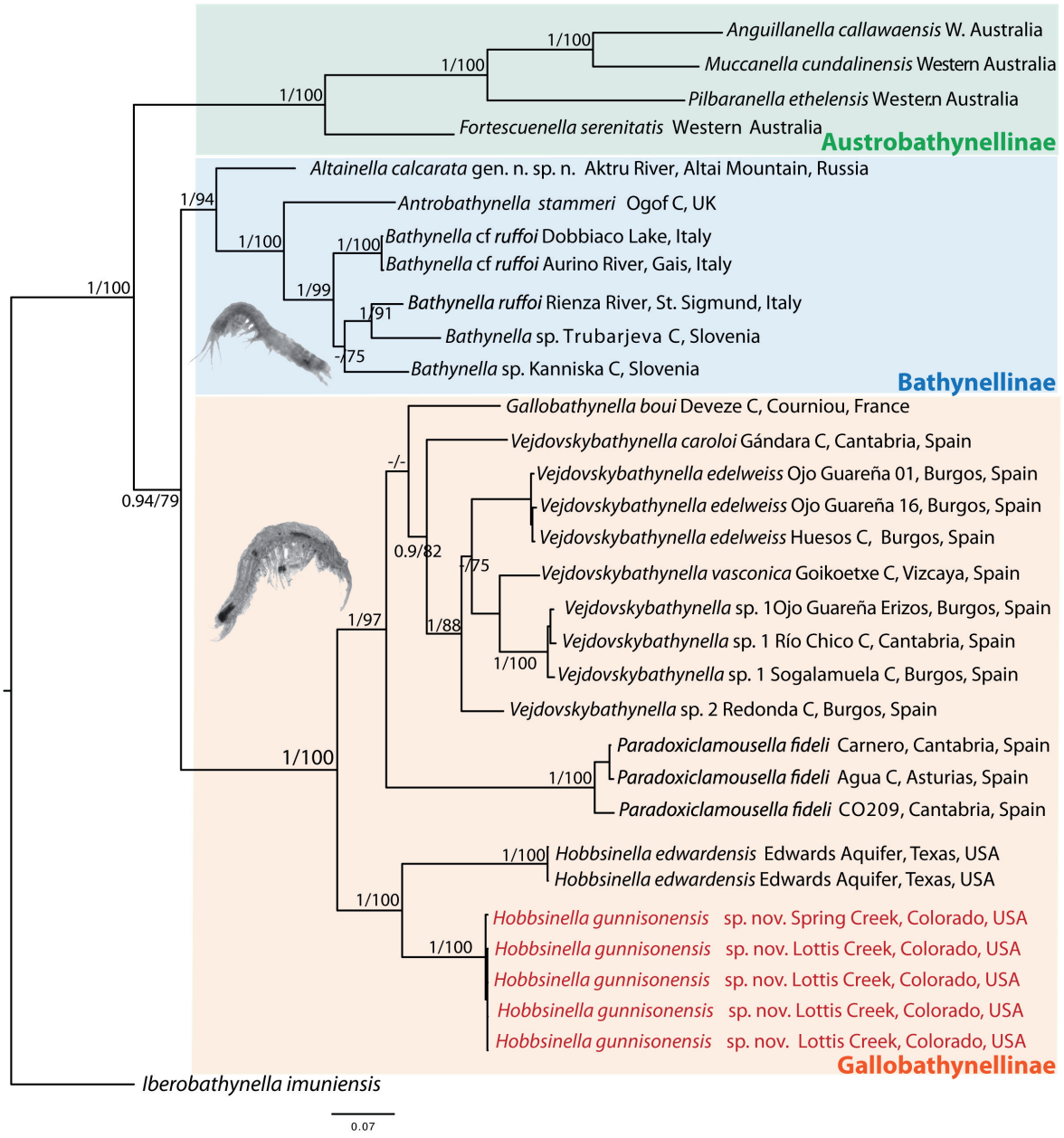

Fig. 5. Phylogenetic relationships among the species of the family Bathynellidae Grobben, 1904 included in this study. The Bayesian phylogenetic tree based on COI and 18S. *Hobbsinella gunnisonensis* Camacho & Taylor sp. nov. is highlighted in red. The same topology was recovered under a Maximum Likelihood approach. Support for each node is represented by the posterior probabilities (PP) resulting from the Bayesian Inference analysis and the bootstrap support values (BS) obtained for the Maximum Likelihood tree (PP/BS). C = cave.

It would be interesting to obtain molecular information of the American species assigned to the genus *Bathynella*, to genetically compare them with the European species of *Bathynella* and see if they form a monophyletic clade. According to Serban (2000), it is unlikely that all species currently assigned to the genus *Bathynella* are really *Bathynella*. Researchers are identifying new morphological characters to further describe the male ThVIII, finding differences that morphologically justify the creation of new genera (e.g., *Camachobathynella* Ranga Reddy, Saik & Totakura, 2015; *Serbanibathynella* Ranga Reddy & Schminke, 2005; *Indobathynella* Ranga Reddy & Totakura, 2012; *Paradoxiclamousella*; *Hobbsinella* and *Altainella*).

Future studies of species within the family should incorporate DNA sequences, because as we are seeing with the most recent papers (Camacho *et al.* 2018a, 2018b, 2020, 2021, 2022; Perina *et al*. 2018, 2019a, 2019b, 2022), molecular information can help to resolve phylogeny and relationships amongst species and genera. Diversification within the family appears to be greater than previously thought. In a complex group with morphological homogeneity, such as Bathynellidae, where a few and difficult characters separate species and genera, the use of additional tools, such as molecular data, is fundamental to understand the diversity and the evolutionary patterns in different countries.

Distribution and paleobiogeography

Until the year 2000 only four species of the family Bathynellidae were known in North America, in two genera, *Bathynella* (*B. riparia* Pennak & Ward,1985, Fig. 1A; *B. fraterna* Cho & Kim, 1997 and *B. germanitas* Cho & Kim, 1997) and *Pacificabathynella* (*P. sequoiae* Schminke & Noodt, 1988) from Colorado and California. Three new species described in 2009 from Montana and one from Alaska (Camacho *et al.* 2009, 2016) expanded the distribution range for this family 3000 km further north. In 2018, a new genus from Texas was described (Camacho *et al*. 2018b). With the description of *H. gunnisonensis* sp. nov. collected from Colorado, the distributional range of the genus is extended more than 1300 km. The two species of *Hobbsinella* also occur in very different drainage basins on opposite sides of the continental divide (Fig. 1A–B).

Numerous North American species have been collected but not formally described: Noodt (1974) reported bathynellids from California and Pennak & Ward (1985) reported bathynellids in the states of Montana, Wyoming, Colorado, Kansas, Oklahoma, Indiana, Ohio and Georgia. These reports show that there is a lot of work to be done on the North American Bathynellidae, and that the diversity described so far is only the 'tip on the iceberg'. It would be very interesting to discover whether the new collections of Bathynellacea were preserved in 100% etanol and refrigerated to be able to obtain DNA sequences even years after their collection and thus would be really useful in future studies.

Within Gallobathynellinae the genera *Vejdovskybathynella*, *Paradoxiclamousella*, *Gallobathynella*, and *Hobbsinella* are distributed in Europa (Spain, France, Germany, Switzerland and Italy) and North America (Texas and Colorado) (Camacho 2019). This subfamily is distributed across two continents with presumed Laurentian origins. Continental drift subdivided the area in the Early Triassic (245 to 205 Ma; Golonka 2007), when the separation of the Iberian Peninsula from North America started (Yilmaz *et al*. 1996). The genera *Antrobathynella* and *Altainella*, are distributed in Eurasia together with some undescribed species of *Bathynella* from Italy and Slovenia. Unfortunately, many species morphologically identified as *Bathynella* from different continents do not have sequenced data to support their position in the phylogeny, therefore the distribution of this genus is uncertain.

The study of the palaeobiogeography of Bathynellidae is difficult, due to their very ancient origins and lack of surface relatives (Coineau & Camacho 2013) and because of their morphological homogeneity. There are no fossils or surface bathynellids discovered so far, so their present-day ranges are influenced by a combination of more or less restricted habitats, lifestyles, and biogeographical patterns reflecting

ancient hydrology. Plate tectonics appears to be the major vicariant process that influenced their evolutionary history at a world-wide scale (Coineau & Camacho 2013). The new species from Colorado described here belongs to a genus described on the other side of the continental divide (Fig. 1A–B) (Texas), and DNA confirmed the sister relationship between the two species. So the actual distribution means that they probably have a common ancestor that was widespread in the past, perhaps with Pangean or Laurentian distributions.

Acknowledgements

We thank Dr Eric Leonard (Colorado College, Colorado Springs, Colorado) for providing data used to produce the map. Financial support was provided by the Hevey Family Fund for Student Research and the Summer Faculty Student Collaborative Research Grant (both grants from Colorado College, Colorado Springs, Colorado, USA). Colorado College's Department of Organismal Biology & Ecology generously provided access to equipment and facilities. Additional support was provided by two USA nonprofits, the Cave Conservancy Foundation and the Subterranean Ecology Institute. We gratefully acknowledge C. Puch who helped us various ways. This work was supported by project PID2019-110243GB-100 by the MICINN/FEDER. P. Mas-Peinado is supported by Postdoctoral Fellowship Margarita Salas CA4/RSUE/2022-00171.

Disclosure statement

No potential conflict of interest is reported by the authors.

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Manuscript received: 23 March 2022
Manuscript accepted: 23 August 2023
Published on: 18 September 2023
Topic editor: Magalie Castelin
Section editor: Fabio Stoch
Desk editor: Pepe Fernández