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Title: Morphology, phylogenetic position, and mating behaviour of a new Mesobiotus
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Year: 2023

Version: Published version

Please cite the original version:

Vecchi, M., McDaniel, J. L., Chartrain, J., Vuori, T., Walsh, E. J., & Calhim, S. (2023). Morphology,
phylogenetic position, and mating behaviour of a new Mesobiotus (Tardigrada) species from a
rock pool in the Socorro Box Canyon (New Mexico, USA). European Zoological Journal, 90(2),
708-725. https://doi.org/10.1080/24750263.2023.2263033

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Morphology, phylogenetic position, and mating behaviour of a new *Mesobiotus* (Tardigrada) species from a rock pool in the Socorro Box Canyon (New Mexico, USA)

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Abstract

Tardigrades have been recorded from a variety of habitats including mosses, lichens, leaflitter, streams, and marine sediments; however, reports from rock pools are still scarce. Rock pools across the world are known to host diverse invertebrate communities and endemisms are common. We provide the description by integrative taxonomy of *Mesobiotus huecoensis* **sp. nov.**, found in the sediment of an ephemeral rock pool in Box Canyon Recreational Area in New Mexico (USA), and placed it in the *M. montanus* morphogroup based on the presence of eggs with hemispherical processes. This new species has elongated claws, particularly on the fourth pair of legs. Elongated claws are typical of freshwater tardigrades, and could represent an adaptation that allows the new species to better move in the substrate when the rock pool is fully inundated. We also provide information on the sperm morphology and mating behaviour of this new species. The finding of this new species highlights the importance of ephemeral rock pools for the discovery of new taxa and the need for their study and conservation.

http://zoobank.org/urn:lsid:zoobank.org:pub:2EE663A1-684B-4BE9-A11A-AD40B46802A2

Keywords: Tardigrades, new species, *Mesobiotus*, huecos, sperm morphology

Introduction

Tardigrades, a phylum of microinvertebrates that includes over 1400 species (Degma & Guidetti 2023), are water-dependent animals that need at least a film of water to survive (Nelson et al. 2018). However, many species have the ability to enter a state called cryptobiosis, where they can resist harsh conditions, such as drying out or freezing (Rebecchi et al. 2007; Hengherr & Schill 2018; Schill & Hengherr 2018). Due to this ability, tardigrades can be found in a wide range of environments including terrestrial,

freshwater, and marine habitats all over the world (Nelson et al. 2018). As a result of their small size and cryptobiotic capabilities, tardigrades colonize a variety of environments, ranging from limno-terrestrial (leaf litter, soil, mosses, lichens) to aquatic habitats (periphyton, sediment) (Nelson et al. 2018) and climatic conditions from glaciers (Zawierucha et al. 2016) to deserts (Darby & Neher 2012).

Tardigrade records in ephemeral freshwater rock pools are extremely limited, having been recorded only a few times in the scientific literature (De Vries

1996; Koste 1996; Spencer et al. 1999; Jocqué et al. 2007; Boix et al. 2016; Velasco-González et al. 2020). However, the potential of this particular habitat for hosting rich and diverse tardigrades communities is evident from recent studies (Vecchi et al. 2022), where a new endemic species from a globally rare genus was discovered (Vecchi et al. 2023).

The family Macrobiotidae is a group of limno-terrestrial tardigrades, within which 14 distinct genera have been recognized so far. However, most of the species' diversity in this family are found in only four genera (*Macrobiotus*, *Mesobiotus*, *Minibiotus*, and *Paramacrobiotus*) (Degma & Guidetti 2023). These genera have been historically recognized as informal species groups within the genus *Macrobiotus*, but were later elevated to the genus rank (Schuster et al. 1980; Guidetti et al. 2009; Vecchi et al. 2016). The genus *Mesobiotus* is the focus of this study and currently comprises 75 nominal species, four of which are designated as *nomina inquirenda*. The genus was erected by Vecchi et al. (2016) and supported by morphological and genetic data. Subsequent studies have shown that the genus is monophyletic, but there is no support for the two traditionally recognized species groups in the genus (*harmsworthi* group and *furciger* group). These two species groups are characterized by distinctive morphologies of the eggs processes (dichotomous branching on the tip of the process present in the *furciger* group and absent in the *harmsworthi* group). However due to their lack of reciprocal monophyly, Short et al. (2022) proposed to abolish the use of these two informal groupings. Stec (2022) argued instead to maintain the usage of these morphogroups as they are useful for taxonomists and name-users in aiding identification and communication regarding taxa. Stec (2022) provided new explicit and clear definitions of the *harmsworthi* and *furciger* morphogroups and proposed the institution of an additional *montanus* morphogroup (defined by the presence of dome-shaped egg processes).

Here we describe, by means of integrative taxonomy, a new species of *Mesobiotus* belonging to the *montanus* morphogroup from ephemeral freshwater rock pools from the northern portion of the U.S. Chihuahuan Desert. In addition, we provide data on its sperm morphology and mating behaviour.

Materials and methods

Study area

The Box Canyon Recreational Area is 18 km southwest of the city of Socorro, NM. It is managed by New Mexico's Bureau of Land Management and covers an area of 2.6 km² with an maximum elevation of 1800 m. There are five cliffs that surround the canyon, and the origin of the rock is volcanic rhyolite (New Mexico Energy, Minerals, and Natural Resources Department). Throughout these rock formations there are many small spatially separated temporary rock pools found near canyon's floor and on top of the surrounding cliffs. The area's vegetation is a mix of Chihuahuan Desert canyon shrubland and Juniper Pinyon Woodlands (McDaniel 2022). Freebird rock pool (Figure 1; created with the software ArcGis Pro 3.1 using maps from Jornada Basin Spatial Data Laboratory 2006; Esri 2017) is located on top of one rocky plateau. It is a shallow rock pool of approximately 1.25 m in maximum length and 1 m in maximum width, with a depth of 4 cm. The rock pool fills during monsoonal rains and is hydrologically connected to a several rock pools in the drainage basin. When dried, there is a blackish algal/biofilm mat in part of the pool.

Sampling and samples processing

A dry sample was taken from the sediment surface (~2.5 cm of thickness) with a clean trowel. The sample was kept desiccated until processing. Tardigrades extraction was performed with the Ludox protocol recommended by Bartels and Nelson (2006) with the modifications by Vecchi et al. (2022) but without the boiling water killing step.

Microscopy and imaging

Specimens for light microscopy were mounted on microscope slides in a small drop of Hoyer's (~200 mg) medium, secured with a cover slip (22 × 22 mm) and dried at 60°C for a week. Additional individuals were stained with Orcein (Bertolani 1971) to identify males and the sperm maturation pattern.

Slides were examined under a Leica DMLB light microscope with phase contrast (PCM), associated with a digital camera (5440 × 3648 pix). For structures that could not be satisfactorily focused on a single light microscope photograph, a stack of 2–5 images were taken with an equidistance of ca. 0.2 μm and assembled manually into a single deep-focus image in GIMP v.2–10 (GIMP Development Team 2019).

Specimens for Scanning Electron Microscopy (SEM) were asphyxiated for 30 minutes at 60°C and subjected to an ascending concentration gradient of ethanol (20%, 50%, 70%, 90%, 95%, 100%, 100%) for 15 minutes each. The specimens were critical point dried in CO_2, mounted on stubs, and coated with gold. The SEM observations were carried out with a Nova Nano SEM 450 (FEI
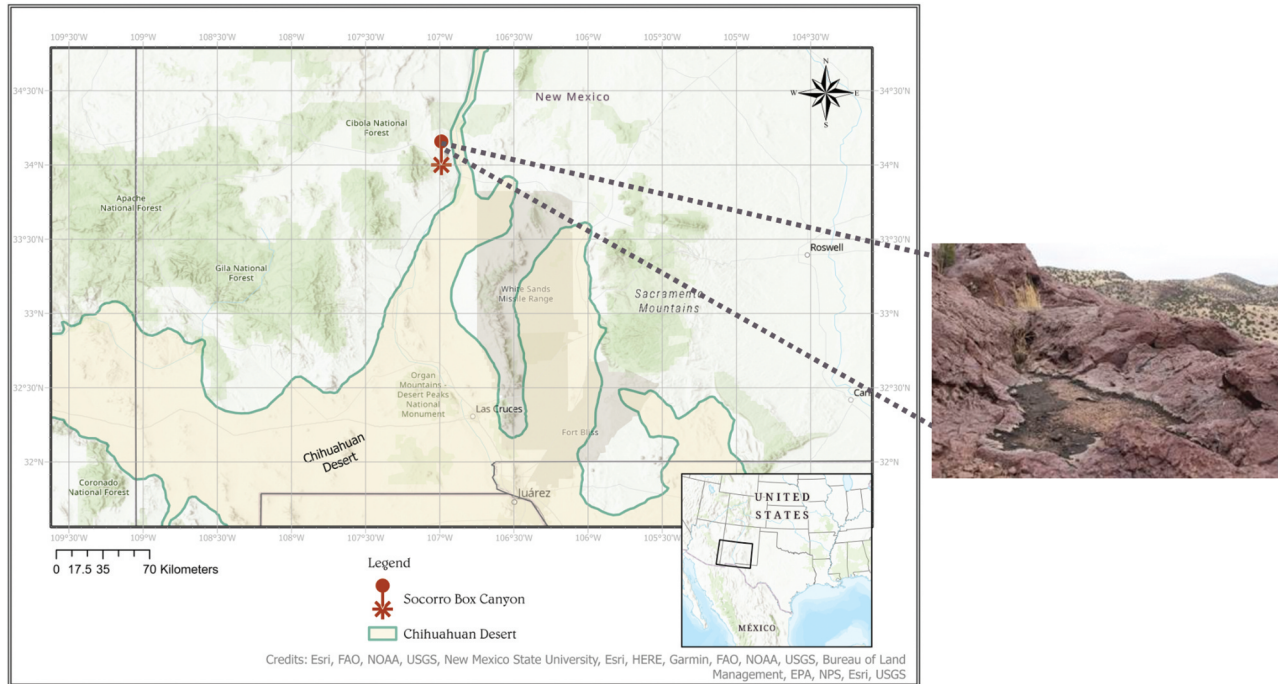
Figure 1. Type locality of *Mesobiotus* sp. nov, Freebird rock pool, Socorro Box Canyon located in near Socorro, New Mexico. Map was made using ArcGIS pro 3.1.1 (ESRI 2017) with Chihuahuan Desert boundary from Jornada Basin Spatial Data Laboratory (2006).

Company—Oxford Instruments, 1536 × 1103pix), available at the "Centro Interdipartimentale Grandi Strumenti" at University of Modena and Reggio Emilia (Modena, Italy).

All figures were assembled in Figure J (Mutterer & Zinck 2013).

Sperm staining and measuring

One male individual (with moving sperm in the gonad) was dissected on a polylysinated slide in 5 µl of 0.1X PBS (Phosphate Buffer Saline) with tungsten needles to release the sperm. The slide was incubated for 10 minutes at room temperature (21°C) in a humidity chamber and then fixed with 50 µl of 1% formaldehyde in 0.1X PBS. After fixing, the solution was removed and the slide was left to air-dry. Staining was performed by adding 32 µl of staining solution (10 µM Phalloidin-TRITC and 0.25% Triton-X100 in 0.1X PBS) for 1 hour in the dark at room temperature. Staining solution was then quickly rinsed with distilled water and replaced with 15 µl of Fluoromount mounting medium with 40 µg/ml Hoechst 33,342. A glass coverslip was then applied and sealed with nail polish. The slide was image 30 minutes after preparation on a Leica S18 Falcon confocal microscope. Phalloidin-TRITC stains actin (acrosome, midpiece, and tail) and was imaged with excitation

at 551 nm and emission at 570-750 nm with an image size of 2048 × 2048 px. Hoechst 33,342 stains the chromatin in the nucleus and was imaged with excitation at 405 nm and emission at 410-480 nm. A 4.5 µm Z-stack of ten equidistant focal planes was acquired. The focal planes were combined by using a maximum intensity Z projection in ImageJ (Schneider et al. 2012), and the sperm components were measured with the same software. The raw sperm measurements are provided in SM.02.

Morphometrics and morphological nomenclature

All measurements are given in micrometers (µm). Structures were measured only if their orientation was suitable. Body length was measured from the anterior extremity to the posterior end of the body, excluding the hind legs. Buccal tube length and the level of the stylet support insertion point were measured according to Pilato (1981). The *pt* index is the ratio of the length of a given structure to the length of the buccal tube (Pilato 1981). Measurements of buccal tube widths, heights of claws and eggs, as well as the terminology used to describe the Oral Cavity Armature (OCA) and eggshell morphology follow Kaczmarek et al. (2020). Morphometric data were handled using the "Parachela" ver. 1.7 template available from the Tardigrada Register. The

raw morphometric data are provided as Supplementary Materials (SM.01).

Thorpe's normalization of morphometric data was performed according to Bartels et al. (2011) with the R script provided by Vecchi and Stec (2021). The results of Thorpe's normalization are provided in SM.03.

Tardigrade taxonomy follows Bertolani et al. (2014), Stec (2022) and Stec et al. (2021).

Comparative material

Photographs of eggs of members of the *Mesobiotus montanus* morphogroups were obtained from the Pilato & Binda collection at the University of Catania, Catania, Italy (*M. mottai* type series 4471 from Antarctica), the Maucci collection hosted at the University of Modena and Reggio Emilia, Modena, Italy (*M. lusitanicus* type series 11,242 from Portugal and *M. montanus* from Italy), and the Tardigrade collection of the Institute of Systematics and Evolution of Animals (Polish Academy of Sciences), Krakow, Poland (*M. peterseni* GL.002 from Greenland).

Genotyping

DNA was extracted from individual animals following a Chelex® 100 resin (BioRad) extraction method by Casquet et al. (2012) with modifications described in detail in Stec et al. (2020). Carcasses were recovered and mounted as hologenophores (voucher codes provided in the new species description section). We attempted to sequence four DNA fragments, three nuclear (18S rRNA, 28S rRNA, ITS2) and one mitochondrial (COI). All fragments were amplified and sequenced according to the primers and protocols described in Stec et al. (2020). Sequencing products were read with an ABI 3130xl sequencer at the Department of Biological and Environmental Sciences (University of Jyväskylä, Finland).

Phylogenetic analysis

The multilocus phylogenetic analysis was conducted using concatenated 18S rRNA + 28S rRNA+ITS-2 +COI sequences. We used the same dataset as in Stec (2022) (Table I), with the addition of the sequences from the new species.

The 18S rRNA, 28S rRNA and ITS-2 sequences were aligned using MAFFT ver. 7 (Katoh 2002; Katoh & Toh 2008) with the G-INS-i method (thread = 4, threadtb = 5, threadit = 0, reorder, adjust direction, any symbol, max iterate = 1000, retree 1, global pair input). The COI sequences were aligned according to their amino acid sequences (translated using the invertebrate mitochondrial code) with the MUSCLE algorithm (Edgar 2004) in MEGA7 with default settings (i.e., all gap penalties = 0, max iterations = 8, clustering method=UPGMB, lambda = 24). Alignments were visually inspected and trimmed in MEGA7. Sequences were concatenated with the R package "concatipede" v1.0.0 (Vecchi & Bruneaux 2021).

Model selection was performed for each alignment partition (6 in total: 18S rRNA, 28S rRNA, ITS-2 and three COI codons) with PartitionFinder2 (Lanfear et al. 2016).

Bayes inference (BI) phylogenetic reconstruction was done with MrBayes v3.2.6 (Ronquist et al. 2012). Two runs with one cold chain and three heated chains were run for 20 million generations with a burn-in of 2 million generations, sampling a tree every 1000 generations. Posterior distributions were checked with Tracer v1.7 (Rambaut et al. 2018). MrBayes input file with the input alignment is available as Supplementary Materials (SM.04).

The phylogenetic tree was visualized with FigTree v1.4.4 (Rambaut 2007) and the image was edited with Inkscape 0.92.3 (Inkscape Project 2020). The complete phylogenetic tree is available in SM.05.

Mating behaviour observation

Individual tardigrades were kept isolated for at least one week in wells of a 24-wells plate filled with mineral water at 21°C. The animals were fed *ad libitum* with algae (*Chlorella* sp. and *Chlorococcum hypnosporum)* and nematodes (*Panagrellus pycnus*). Five pairs of sexually mature individuals (females with large and evident oocytes in the gonad and males with gonad filled with motile sperm) were used. Each pair was placed in a well of a Ibidi μ-Slide 15 Well 3D slide (4 mm on diameter), with the bottom covered with 10 μl of 1% agar in mineral water. Each couple was then recorded under an inverted microscope Carl Zeiss Cell Observer HS (2009/ 2009) linked to a Zeiss AxioCam MRm (1388 × 1040 pix, pix size 6.45 μm) for at least 20 minutes. Video were analysed with the software BORIS (Friard & Gamba 2016) using the following ethogram: (i) contact: contact between both individuals (touching of any body part at the exclusion of cloacas); (ii) follow: male follows the female; (iii) mount: male aligns his cloaca with that of the female; (iv) touch: male touches the female cloaca; (v) ejaculation: visible sperm is released by the male. Plots of the tardigrade behaviour observed in each video are provided in SM.06.

Table I. Sequences used for phylogenetic analysis.

Species	18S rRNA	28S rRNA	ITS-2	COI	Source
Mesobiotus huecoensis **sp. nov.**	OQ756249			OQ756246	This study
	OQ756248			OQ756247	This study
Mesobiotus diegoi Stec, 2022	OP142527	OP142520	OP142514	OP143858	Stec (2022)
	OP142526	OP142521	OP142515	OP143857	Stec (2022)
Mesobiotus maklowiczi Stec, 2022	OP142525	OP142518		OP143855	Stec (2022)
	OP142524	OP142519		OP143856	Stec (2022)
Mesobiotus peterseni (Maucci, 1991)	OP142528	OP142522	OP142516	OP143859	Stec (2022)
	OP142529	OP142523	OP142517	OP143860	Stec (2022)
Mesobiotus ethiopicus Stec & Kristensen, 2017	MF678793	MF678792	MN122776	MF678794	Stec and Kristensen (2017)
Mesobiotus datanlanicus Stec, 2019	MK584659	MK584658	MK584657	MK578905	Stec (2019)
Mesobiotus dilimanensis Itang et al., 2020	MN257048	MN257049	MN257050	MN257047	Itang et al. (2020)
Mesobiotus philippinicus Mapalo et al., 2016	KX129793	KX129794	KX129795	KX129796	Mapalo et al. (2016)
Mesobiotus insanis Mapalo et al., 2017	MF441488	MF441489	MF441490	MF441491	Mapalo et al. (2017)
Mesobiotus hilariae Vecchi et al., 2016	KT226070			KT226108	Vecchi et al. (2016)
Mesobiotus radiatus (Pilato etal., 1991)	MH197153	MH197152	MH197267	MH195147	Stec et al. (2018)
			MH197268	MH195148	Stec et al. (2018)
Mesobiotus romani Roszkowska et al., 2018	MH197158	MH197151	MH197150	MH195149	Roszkowska et al. (2018)
Mesobiotus harmsworthi (Murray, 1907)	MH197146	MH197264	MH197154	MH195150	Kaczmarek et al. (2018)
				MH195151	Kaczmarek et al. (2018)
Mesobiotus occultatus Kaczmarek et al., 2018	MH197147		MH197155	MH195152	Kaczmarek et al. (2018)
Mesobiotus furciger group species NO	MH197148	MH197265	MH197156	MH195153	Kaczmarek et al (2018)
Mesobiotus harmsworthi group species RU	MH197149	MH197266	MH197157	MH195154	Kaczmarek et al. (2018)
Mesobiotus fiedleri Kaczmarek et al., 2020	MH681585	MH681693	MH681724	MH676056	Kaczmarek et al. (2018)
Mesobiotus anastasiae Tumanov, 2020	MT903468	MT903612	MT903470	MT904513	Tumanov (2020)
Mesobiotus skorackii Kaczmarek et al., 2018		MW680636		MW656257	Kayastha et al. (2021)
Mesobiotus imperialis Stec, 2021	OL257854	OL257866		OL311514	Stec (2021)
	OL257855	OL257867		OL311515	Stec (2021)
Mesobiotus marmoreus Stec 2021	OL257856	OL257868	OL257861	OL311516	Stec (2021)
	OL257857	OL257869	OL257862	OL311517	Stec (2021)
	OL257858	OL257870	OL257863	OL311518	Stec (2021)
Mesobiotus cf. *barabanovi*	MN310392	MN310388	MN310390	MN313170	Kaczmarek et al. (2020)
Mesobiotus sp. Macro07_042	MW751942			MW727957	Short et al. (2022)
Mesobiotus cf. *furciger* Macro06_296	MW751936			MW727958	Short et al. (2022)
Mesobiotus cf. *furciger* Macro06_310	MW751937			MW727961	Short et al. (2022)
Mesobiotus cf. *furciger* Macro06_313	MW751939			MW727960	Short et al. (2022)
Mesobiotus cf. *furciger* CC_MF_4	MW751949			MW727933	Short et al. (2022)
Mesobiotus cf. *furciger* ABDC_MF_3	MW751944			MW727932	Short et al. (2022)
Mesobiotus cf. *furciger* KPRI_MF_1	MW751962			MW727934	Short et al. (2022)
Mesobiotus cf. *furciger* HMI_MF_1	MW751957			MW727941	Short et al. (2022)
Mesobiotus cf. *furciger* EBNI_MF_2	MW751952			MW727937	Short et al. (2022)
Mesobiotus cf. *furciger* EBNI_MF_4	MW751954			MW727938	Short et al. (2022)
Mesobiotus cf. *furciger* PSAI_MF_2	MW751967			MW727939	Short et al. (2022)
Mesobiotus cf. *furciger* Macro06_162	MW751934			MW727955	Short et al. (2022)
Mesobiotus cf. *furciger* Macro06_171	MW751935			MW727956	Short et al. (2022)
Mesobiotus cf. *furciger* JN07_MF_1	MW751959			MW727951	Short et al. (2022)
Mesobiotus cf. *furciger* JN07_MF_4	MW751960			MW727953	Short et al. (2022)
Mesobiotus cf. *furciger* JN07_MF_8	MW751961			MW727947	Short et al. (2022)
Mesobiotus cf. *furciger* FN01_MF_6	MW751955			MW727945	Short et al. (2022)
Macrobiotus kamilae Coughlan & Stec, 2019	MK737070	MK737064	MK737067	MK737920	Coughlan and Stec (2019)
				MK737921	Coughlan and Stec (2019)
Macrobiotus hannae Nowak & Stec, 2018	MH063922	MH063924	MH063923	MH057764	Nowak and Stec (2018)

Results

Phylogenetic reconstruction

The BI phylogenetic reconstruction (Figure 2) of the genus *Mesobiotus* yielded a generally poorly supported tree, but with a topology that is still in agreement with that of Stec (2022). The new species is placed in an unresolved clade comprising *M. harmsworthi* (the type species of the genus), *M. occultatus*, *M. aff. Harmsworthi* RU017, and *M. peterseni*.
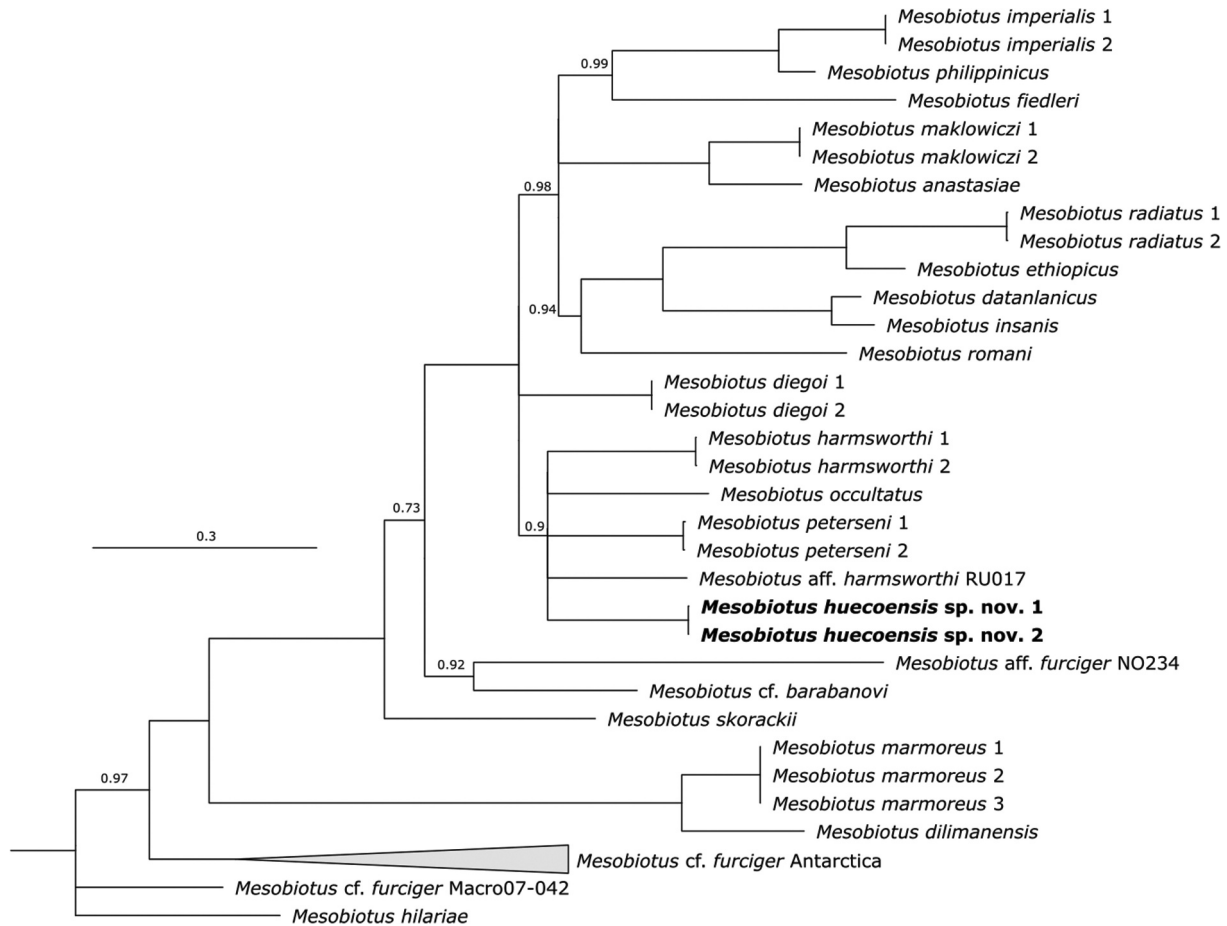
Figure 2. Bayesian phylogenetic reconstruction of the genus *Mesobiotus*. Numbers above nodes indicate bayesian posterior probability (pp) (shown when pp = 1). Nodes with pp < 0.70 were collapsed. The new described species is highlighted in bold. Scale bar indicates the number of substitutions/site. Outrgroup taxa are not shown. For a complete version of the phylogenetic reconstruction including nodes with pp < 0.70 and outgroups see SM.03.

Taxonomic account

Phylum: Tardigrada Doyère, 1840
Class: Eutardigrada Richters, 1926
Order: Parachela Schuster, Nelson, Grigarick and Christenberry, 1980
Superfamily: Macrobiotoidea Thulin, 1928 (in Marley et al., 2011)
Family: Macrobiotidae Thulin, 1928
Genus: *Mesobiotus* Vecchi, Cesari, Bertolani, Jönsson, Rebecchi and Guidetti, 2016

Mesobiotus huecoensis Vecchi, McDaniel & Walsh 2023 sp. nov.

https://urn:lsid:zoobank.org:act:D935E484-2E38-47E4-BF84-BD9F9E12E2B0

(Figures 1–6; Tables II–IV; SM.01–3)

Material examined: 29 animals and 19 eggs mounted on microscope slides in Hoyer's medium, two animals stained with Orcein, one egg examined by SEM, one specimen was processed for sperm measurement and two specimens were processed for DNA sequencing and the carcasses were mounted on microscope slides in Hoyer's medium.

Type locality: 34°00'02.9"N 106°59'22.6"W; 1730 m asl: Freebird rock pool, Socorro Box Canyon, New Mexico (U.S.); sediment from ephemeral rock pool; collected 5th January 2022.

Table II. Measurements [in μm] and pt values of selected morphological structures of individuals of *Mesobiotus huecoensis* **sp. nov**.; specimens mounted in Hoyer's medium.

CHARACTER	N		RANGE				MEAN		SD		Holotype	
	μm	pt	μm		pt		μm	pt	μm	pt	μm	pt
Body length	29	24	257 –	682	877 –	1218	462	1080	128	94	604	1218
Buccopharyngeal tube												
Buccal tube length	24		29.2 –	53.1	–		41.1	–	8.9	–	49.6	–
Stylet support insertion point	23	23	16.8 –	38.0	44.7 –	76.2	29.3	71.7	7.2	6.1	37.0	74.6
Buccal tube external width	24	24	3.7 –	32.4	11.4 –	61.0	6.4	15.0	5.7	9.8	7.1	14.3
Buccal tube internal width	24	24	2.2 –	5.3	7.5 –	10.0	3.5	8.4	0.9	0.6	4.1	8.3
Ventral lamina length	23	23	8.2 –	36.1	15.4 –	74.5	26.1	62.9	7.4	11.1	31.5	63.5
Placoid lengths												
Macroplacoid 1	24	24	2.4 –	7.3	8.2 –	14.1	4.8	11.3	1.8	2.1	7.0	14.1
Macroplacoid 2	24	24	2.3 –	6.0	7.8 –	12.3	4.2	10.1	1.3	1.2	5.9	11.9
Macroplacoid 3	24	24	2.6 –	7.3	8.8 –	13.7	4.7	11.2	1.6	1.5	6.2	12.5
Microplacoid	24	24	1.3 –	4.9	4.3 –	9.4	2.8	6.6	1.1	1.5	4.0	8.1
Macroplacoid row	24	24	9.0 –	22.3	30.7 –	42.0	15.5	37.1	4.6	3.5	20.7	41.7
Placoid row	24	24	11.4 –	28.2	38.9 –	53.1	19.2	45.8	5.8	4.4	25.1	50.6
Claw I heights												
External base	23	20	3.6 –	10.5	12.3 –	20.5	6.4	15.2	2.1	2.3	6.5	13.1
External primary branch	29	24	9.1 –	19.5	27.8 –	38.0	13.7	32.8	3.3	2.4	16.6	33.5
External secondary branch	28	24	6.1 –	13.8	20.0 –	27.0	10.3	24.9	2.5	2.0	13.1	26.4
External base/primary branch (cct)	23		35.5 –	63.5	–		46.0	–	6.8	–	39.2	–
Internal base	23	20	3.3 –	10.0	10.9 –	19.5	6.0	14.1	2.1	2.5	6.7	13.5
Internal primary branch	29	24	8.1 –	18.6	23.1 –	36.3	13.2	31.2	3.3	2.6	16.0	32.3
Internal secondary branch	29	24	6.1 –	14.2	17.9 –	27.7	9.6	23.0	2.5	2.4	12.9	26.0
Internal base/primary branch (cct)	23		33.8 –	62.0	–		45.3	–	7.7	–	41.9	–
Claw II heights												
External base	24	20	3.3 –	9.9	11.3 –	19.0	6.6	15.8	1.9	2.3	8.4	16.9
External primary branch	28	23	9.5 –	21.1	31.9 –	40.4	14.9	35.5	3.4	2.5	19.1	38.5
External secondary branch	28	23	6.3 –	16.5	19.5 –	32.2	10.7	25.4	2.9	2.7	14.0	28.2
External base/primary branch (cct)	24		32.7 –	54.0	–		43.6	–	5.6	–	44.0	–
Internal base	25	21	3.5 –	9.9	9.6 –	19.3	6.4	14.7	2.0	2.4	7.1	14.3
Internal primary branch	28	23	9.4 –	19.9	30.2 –	38.0	14.4	33.9	3.6	2.1	16.4	33.1
Internal secondary branch	28	23	6.6 –	16.0	22.3 –	31.3	10.9	25.8	3.0	2.3	14.1	28.4
Internal base/primary branch (cct)	25		29.5 –	60.5	–		44.1	–	7.3	–	43.3	–
Claw III heights												
External base	23	20	3.7 –	10.6	9.9 –	20.7	7.1	16.5	2.1	2.9	7.8	15.7
External primary branch	28	23	10.0 –	20.8	32.5 –	42.2	15.4	36.5	3.6	2.4	17.5	35.3
External secondary branch	28	23	5.9 –	41.2	19.5 –	80.8	12.2	28.7	6.4	11.7	14.1	28.4
External base/primary branch (cct)	23		30.6 –	58.4	–		44.6	–	8.0	–	44.6	–
Internal base	20	18	3.8 –	10.8	10.5 –	21.6	6.8	15.8	2.3	3.0	7.9	15.9
Internal primary branch	28	23	8.9 –	21.4	28.0 –	41.7	14.5	34.5	3.9	3.1	17.3	34.9
Internal secondary branch	27	22	6.6 –	16.3	20.3 –	30.7	10.5	25.0	3.0	2.5	13.9	28.0
Internal base/primary branch (cct)	20		31.6 –	58.4	–		46.3	–	7.1	–	45.7	–
Claw IV heights												
Anterior base	24	20	3.7 –	11.8	12.2 –	23.6	7.2	16.8	2.6	3.4	8.8	17.7
Anterior primary branch	28	23	9.0 –	22.9	30.8 –	48.4	17.0	40.3	4.4	4.1	18.5	37.3
Anterior secondary branch	28	23	6.9 –	16.6	21.5 –	32.4	11.6	27.7	3.3	2.8	15.4	31.0
Anterior base/primary branch (cct)	24		29.7 –	51.5	–		41.3	–	6.8	–	47.6	–
Posterior base	24	19	3.9 –	11.0	10.3 –	20.7	7.0	16.2	2.2	2.8	9.8	19.8
Posterior primary branch	28	23	11.3 –	24.8	37.9 –	49.9	17.8	42.5	4.1	3.0	19.8	39.9
Posterior secondary branch	28	23	6.6 –	16.6	22.3 –	31.3	11.8	28.0	3.0	2.5	14.8	29.8
Posterior base/primary branch (cct)	24		24.6 –	49.5	–		38.5	–	6.0	–	49.5	–

Etymology: From the Spanish, "hueco" means rock pool. In reference to the habitat where the new species has been found.

Type depositories: Holotype: slide S2027_SL3 with 5 paratypes, 24 paratypes (on slides S2027_SL1–4, 6), 19 eggs (on slide S2027_SL7) and 1 hologenophore

Table III. Measurements [in μm] of the eggs of *Mesobiotus huecoensis* **sp. nov**.; eggs mounted in Hoyer's medium; process base/height ratio is expressed as percentage.

CHARACTER	N	RANGE			MEAN	SD
Egg bare diameter	15	60.9	–	78.6	70.8	5.1
Egg full diameter	15	74.6	–	92.3	85.9	5.2
Process height	48	2.3	–	6.4	4.8	0.8
Process base width	48	4.6	–	7.5	6.1	0.7
Process base/height ratio	48	93%	–	250%	131%	32%
Inter-process distance	45	0.7	–	4.7	2.6	0.9
Number of processes on the egg circumference	15	21	–	30	26.5	2.8

Table IV. Measurements [in μm] of the sperms of *Mesobiotus huecoensis* **sp. nov**. stained with Phalloidin-TRITC and Hoechst 33,342.

CHARACTER	N	RANGE			MEAN	SD
Acrosome	8	4.1	–	6.2	5.0	0.8
Nucleus	13	14.4	–	16.7	15.5	0.6
Midpiece	13	8.6	–	10.9	9.5	0.7
Tail	10	20.6	–	24.5	22.7	1.2
Total length	7	51.5	–	53.5	52.7	0.6

(Voucher_S2027_Meb.1) are deposited at the Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Sławkowska 17, 31–016, Kraków, Poland, whereas one SEM stub with 1 egg (S2027_Stub1), 5 paratypes (S2027_SL5) and 1 hologenopore voucher (Voucher_S2027_Meb.4) are deposited in the Bertolani collection in the Laboratory of Evolutionary Zoology, Department of Life Sciences, University of Modena and Reggio Emilia, Via Campi 213/d, 41125 Modena, Italy (Slides codes in Bertolani collection: paratypes C5102_S1 and hologenophore voucher C5102_V01).

Animals: (measurements and statistics in Table II, raw morphometric data in SM.01) Body almost transparent in small specimens, whitish in adults (Figure 3a). Eyes present in alive animals and dissolved by Hoyer's medium in approximately 70% of mounted animals. Body cuticle smooth and without pores.

Claws of the *Mesobiotus* type (Figure 3b, c), with a peduncle connecting the claw to the lunula, a basal septum, and well-developed accessory points situated parallel to the primary branch. Claws on legs IV with elongated main branches (Figure 3c). A single continuous cuticular bar with shadowed extensions toward double muscle attachments is present below claws I–III (Figure 3b), while a faintly visible horseshoe shaped structure connects the anterior and posterior lunulae on claws IV (Figure 3c). A fine granulation is present on the external surface and on the internal surface of legs I–III (Figure 3d, e). Granulation is also present on the lateral and dorsal surfaces of legs IV (Figure 3c, f). A cuticular bulge, similar to a pulvinus, is present on the internal surface of legs I–III (Figure 3e). Lunulae under all claws smooth (Figure 3b, c).

Mouth antero-ventral. Bucco-pharyngeal apparatus of the *Macrobiotus* type (Figure 4a), with ventral lamina, and ten small peribuccal lamellae. The oral cavity armature is well-developed and composed of three bands of teeth (Figure 4d, g). The first band of teeth is composed of numerous small granules situated anteriorly in the oral cavity, just behind the bases of the peribuccal lamellae (Figure 4f). The second band of teeth is located between the ring fold and the third band of teeth is composed of ridges parallel to the main axis of the buccal tube that are larger than those in the first band (Figure 4d–g). The teeth of the third band are located within the posterior portion of the oral cavity, between the second band of teeth and the opening of the buccal tube (Figure 4d–g). The third band of teeth is discontinuous and divided into a dorsal and ventral portion. Under PCM, dorsal and ventral teeth are visible as two lateral ridges and one median transverse ridge (Figure 4d–g). Sometimes, the median ridge (both dorsal and ventral) is subdivided in two parts (Figure 4e, g). An additional mucrone can present behind the median part of the ventral third OCA band (Figure 4f). The pharyngeal bulb is subspherical (Figure 4a), with triangular apophyses, three rod-shaped macroplacoids, and a drop-shaped microplacoid placed close to the third macroplacoid Figure 4a–c). The macroplacoid length sequence is 2 < 3 < 1. The first macroplacoid is anteriorly narrowed and the third has a clearly defined subterminal constriction (Figure 4b, c).

Eggs: (measurements and statistics in Table III, raw morphometric data in SM.01) White – yellowish, laid free, spherical in shape, and equipped with evenly spaced processes in the shape of domes (Figures 5-6). Egg surface between the processes
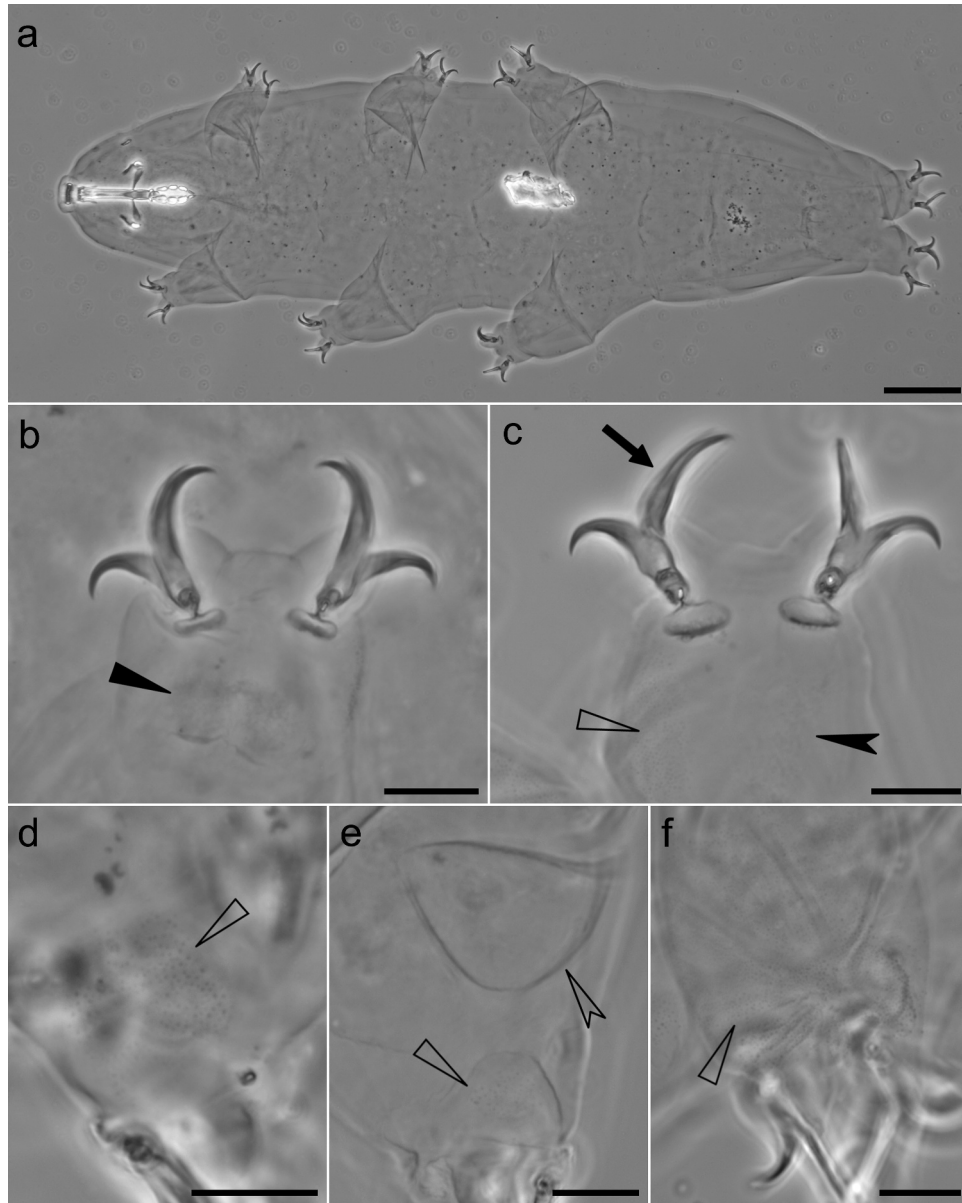
Figure 3. *Mesobiotus huecoensis* **sp. nov**. habitus and leg structures under PCM. (a) Holotype habitus. (b) Claws on leg I from paratype on slide SL1. (c) Claws on leg IV from holotype. (d) Granulation on external side of leg I from paratype on slide SL1. (e) Cuticular bulge on the internal side of leg III from paratype on slide SL1. (f) Granulation on legs IV from paratype on slide SL1. Arrowhead: continuous cuticular bar with with shadowed extensions toward the double muscle attachments. Arrow: flexible primary branch on claws IV. Indented arrowhead: horseshoe-shaped structure connects the anterior and posterior lunulae on claws IV. Empty arrowhead: granulation on legs. Empty indented arrowhead: cuticular bulge on internal leg surface. Images a- e were assembled from multiple focus stacks. Scalebars: 10 μm.

without areolation. In PCM the egg surface between processes appears covered by an irregular reticulum (Figure 5a, d). In SEM, the state is intermediate between pores and reticulum, as nodes and bars are of the same size (or bigger) than pores (Figure 6b, c). Faint dark thickenings, visible under PCM, are present around the bases of the processes (Figure 5d). The labyrinthine layer is visible under PCM as irregular bubbles with usually, but not always, a bigger bubble in the apical part of the process (Figure 5e–j). Pores on the processes surface (mostly concentrated in the bottom half of the processes) are present and visible both in LM (Figure 5c, g) and SEM (Figure 6).
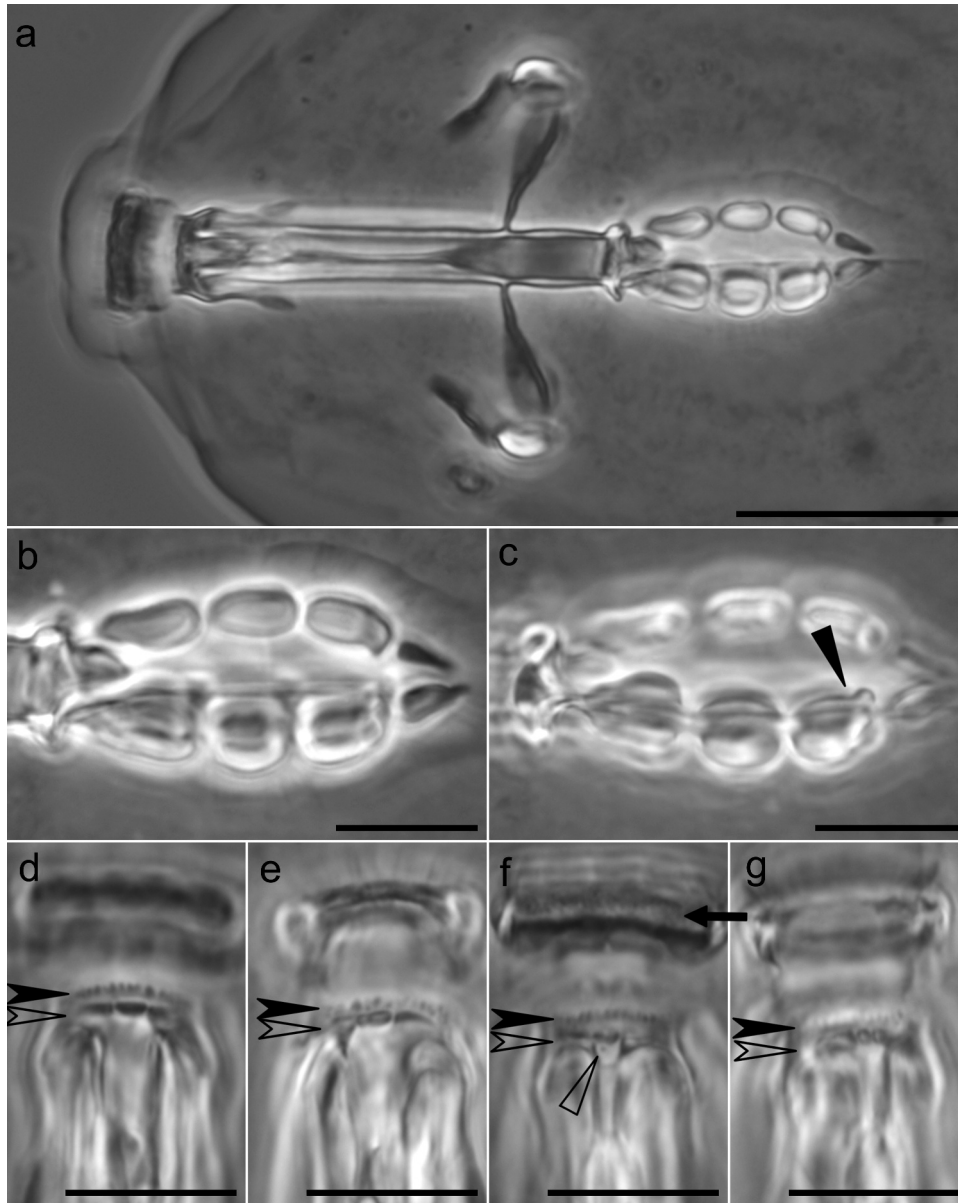
Figure 4. *Mesobiotus huecoensis* **sp. nov**. buccopharyngeal apparatus under PCM. (a) Buccopharyngeal apparatus of the holotype. (b) Dorsal view of the placoids row of the holotype. (c) Ventral view of the placoids row of the holotype. (d) Dorsal part of the oral cavity armature (OCA) of the holotype. (e) Dorsal part of the OCA of a paratype on slide SL1. (f) Ventral part of the OCA of the holotype. (g) Ventral part of the OCA of a paratype on slide SL1.Arrowhead: constriction in the third macroplacoid. Arrow: first (anterior) OCA band. Indented arrowhead: second (middle) OCA band. Empty indented arrowhead: third (posterior) OCA band. Empty arrowhead: mucrone behind the median part of the ventral third (posterior) OCA. Image a was assembled from a multiple focus stack. Scalebars: 10 μm.

Reproduction: The new species is dioecious. In males, the testes, filled with sperm, are clearly visible under PCM after orcein staining (Figure 7a). The new species does not exhibit male secondary sexual dimorphism traits such as lateral gibbosities on legs IV. The presence in the testis of both mature spermatozoa and cell at earlier spermatogenesis stages suggests a continuous pattern of maturation (Rebecchi & Bertolani 1994).

Mating behaviour

The following behaviour and mounting attempts were observed in four out of five mating trials. However, ejaculation was only recorded in one
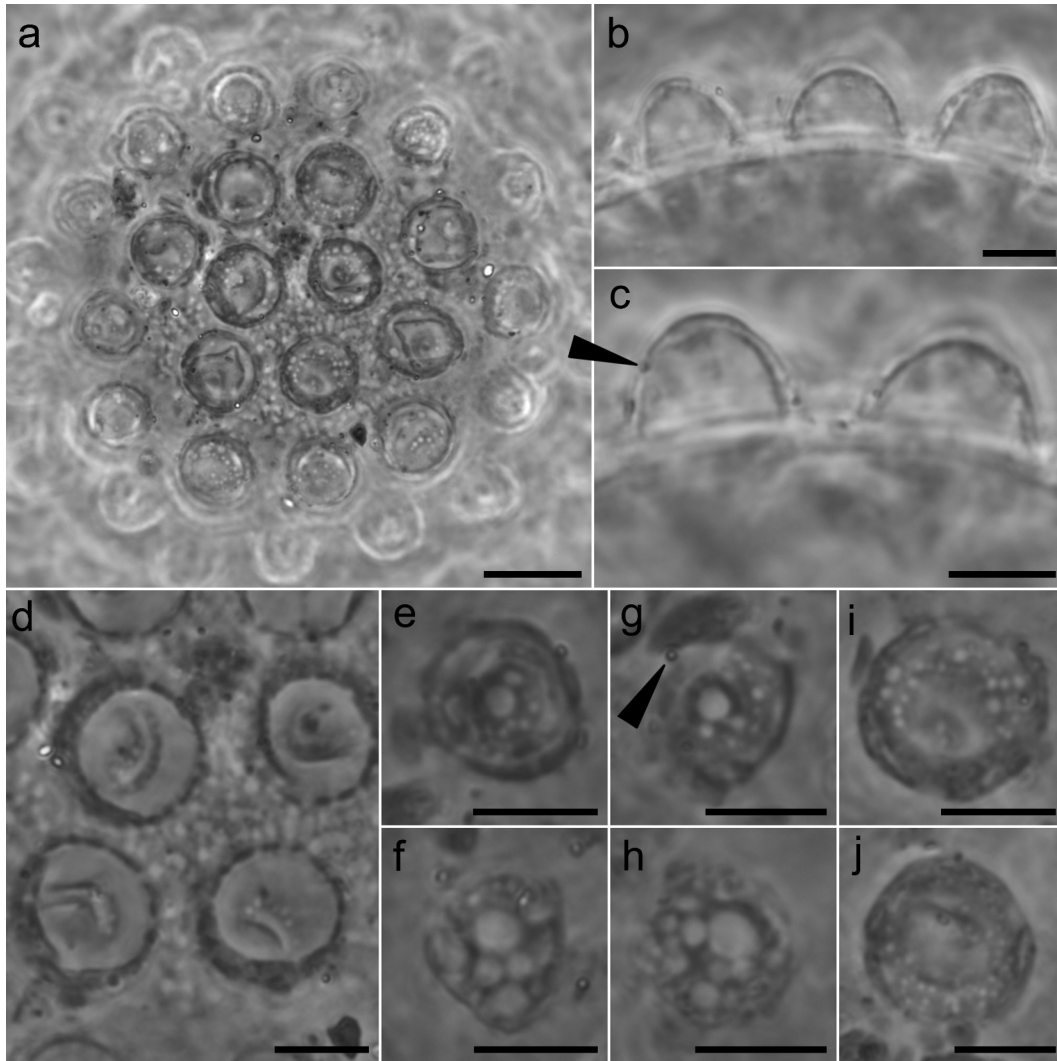
Figure 5. *Mesobiotus huecoensis* **sp. nov**. eggs under PCM. (a) General view of the egg showing rounded processes and reticulation on the chorion. (b, c) Sections of egg processes. (d) Egg chorion covered by a reticulum. (e – j) Dorsal view of individual egg processes showing their variability. Arrowheads: pores on the processes. Image a was assembled from a multiple focus stack. Scalebars: (a) 10 μm, (b – j) 5 μm.

case (SM.07). The mating behaviour sequence of this species is concordant with the observations of Sugiura and Matsumoto (2021a) on another unde-scribed *Mesobiotus* species: tracking (i.e., moving around and physical interaction), touching (i.e., the male touches the female cloaca), standstill (i.e., the female stops moving), and mounting/ejaculation (i.e., the male aligns his cloaca with the female's one and may release sperm). Two distinct sperm release events were observed, both occurring at about one body length distance between male and female indi-viduals and within the first ten minutes of the trial (SM.07).

Sperm: (measurements and statistics in Table IV) Typical morphology of Macrobiotoidea sperm (Figure 7b) (Sugiura & Matsumoto 2021a, 2021b) with an average total length of 52.7 μm [SD 0.6 μm]. Composed of an acrosome (average 5 μm [SD 0.6 μm]), followed by nucleus and midpiece (average 15.5 μm [SD 0.7 μm] and 9.5 μm [SD 0.7 μm], respectively) and tail (average 22.7 μm [SD 1.2 μm]).

DNA sequences: The sequences were obtained for only two (SSU and COI) out of the four molecular markers tested (SSU, LSU, COI and ITS2). LSU and ITS2 failed to amplify.

- **SSU**: Voucher S2027_Meb.1 OQ756248; Voucher S2027_Meb.4 OQ756249
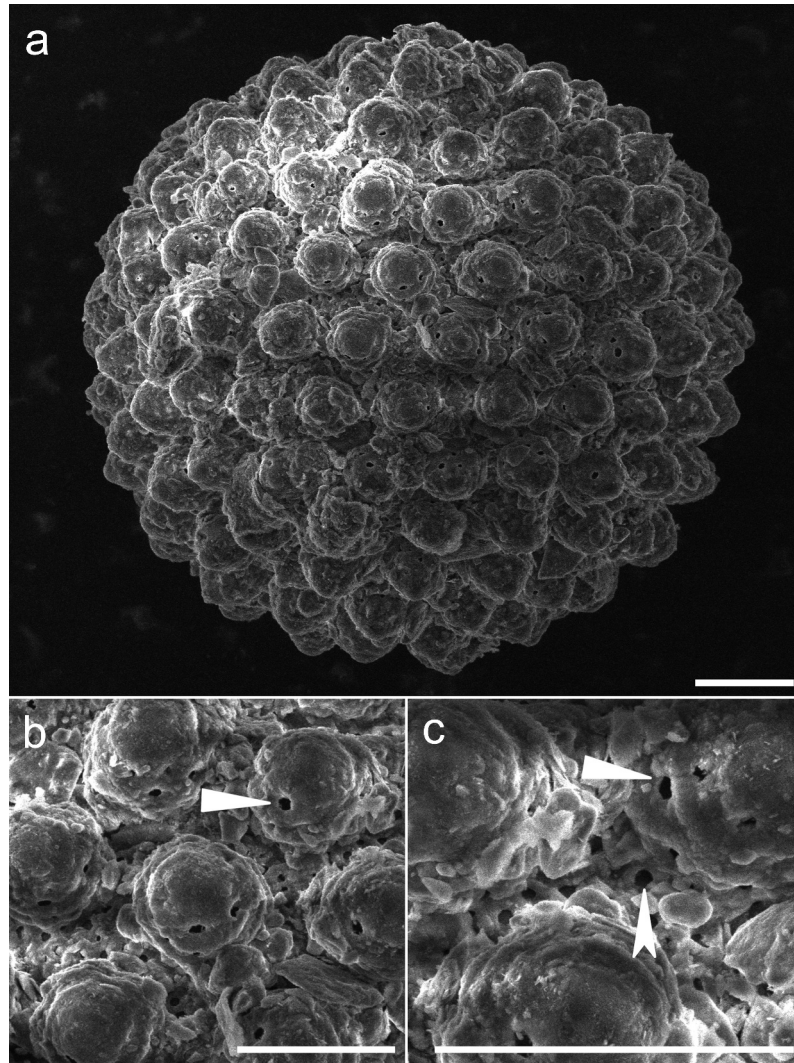- **COI**: Voucher S2027_Meb.1 OQ756246; Voucher S2027_Meb.1 OQ756247

Figure 6. *Mesobiotus huecoensis* **sp. nov**. egg under SEM. (a) *In toto* view. (b) Egg processes showing pores on their lower half. (c) Focus on the reticulated eggshell chorion. Arrowheads: pores on the processes. Indented arrowhead: hole in the chorion reticulum. Scalebars:10 μm.

Additional notes: this species was found with the rotifer *Adineta vaga* (Davis, 1873) and unidentified nematodes.

Differential diagnosis

By having egg processes in the shape of hemispherical or mammillate-like domes, *Mesobiotus huecoensis* **sp. nov**. belongs to the *montanus* morpho-group. The new species differs from all the other species in the *montanus* morpho-group by the elongated primary branch in claws IV (not elongated in all other species). Specifically, the new species differs from:

- *Mesobiotus lusitanicus* (Maucci and Durante Pasa, 1984) by the presence of a labyrinthine layer in the egg processes walls (not visible in *M. lusitanicus*, Figure 8 *vs*. visible in *M. huecoensis* **sp. nov**. Figure 5. Maucci and Durante-Pasa (1984) reported a high variability in the shape *M. lusitanicus* egg processes, however we considered in this differential diagnosis only the egg morphotype that is most similar to the eggs of *M. huecoensis* **sp. nov.**
- *Mesobiotus montanus* (Murray, 1910) by the presence of a labyrinthine layer in the egg processes walls (not visible in *M. montanus*, Figure 8b *vs*. visible in *M. huecoensis* **sp. nov**. Figure 5) and by
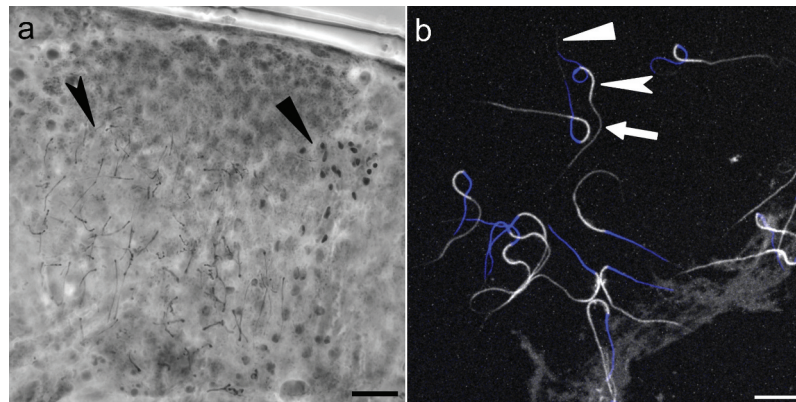
Figure 7. *Mesobiotus huecoensis* **sp. nov**. male gonad and spermatozoa. (a) Male gonad under PCM stained with orcein showing different maturation stages of the male gamets. (b) Stained spermatozoa under Confocal Microscopy, blue represents stained DNA (nucleus), whereas white represents Phalloidin-stained actin (acrosome, midpiece, tail). Black arrowhead: spermatids. Black indented arrowhead: mature spermatozoa. White arrowhead: acrosome. White indented arrowhead: midpiece. White arrow: tail. Scalebars:10 μm.
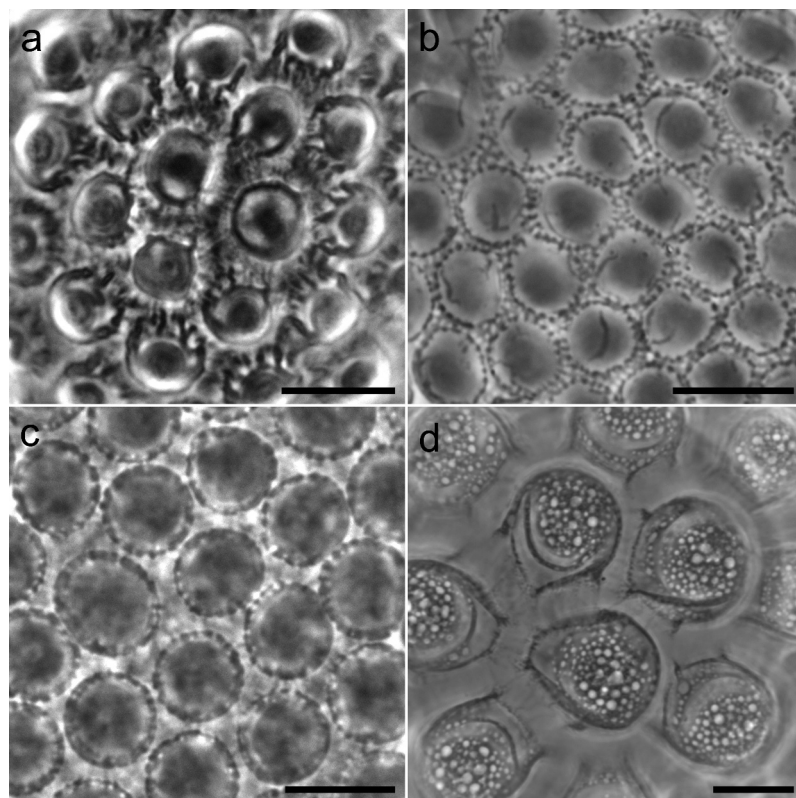

Figure 8. Comparative morphology of *Mesobiotus montanus* morphogroup species under PCM. (a) *M. lusitanicus* from type series. (b) *M. montanus*. (c) *M. mottai* from type series. (d) *M. peterseni*. Scale bars:10 μm.

the spacing between processes (very close by in *M. montanus*, Figure 8b *vs*. spaced in *M. huecoensis* **sp. nov**. Figure 5).

- *Mesobiotus mottai* (Binda & Pilato, 1994) by the presence of a reticulation between the egg processes (not visible in *M. mottai*, Figure 8c *vs*. visible in *M. huecoensis* **sp. nov**.

Figure 5), by the presence of a labyrinthine layer in the egg processes walls (not visible in *M. mottai*, Figure 8c *vs*. visible in *M. huecoensis* **sp. nov**. Figure 5), and by the spacing between processes (very close by in *M. mottai*, Figure 8c *vs*. spaced in *M. huecoensis* **sp. nov**. Figure 5).

- *Mesobiotus peterseni* (Maucci, 1991) by the presence of a reticulation between the egg processes (not visible in *M. peterseni*, Figure 8d *vs*. visible in *M. huecoensis* **sp. nov**. Figure 5), and by more numerous processes on the egg circumference (21 – 30 in *M. huecoensis*, 13 – 15 in *M. peterseni*).

By having elongated primary claw IV branches, *M. huecoensis* is similar *to Mesobiotus altitudinalis* and *Mesobiotus barabanovi*, but differs from:

- *Mesobiotus altitudinalis* (Biserov, 1997/8) by the egg processes shape (domes in *M. huecoensis* **sp. nov**. vs. pointed cones in *M. altitudinalis*).
- *Mesobiotus barabanovi* (Tumanov, 2005) by the egg processes shape (domes *in M. huecoensis* **sp. nov**. vs. pointed cones in *M. barabanovi*), the presence of smooth lunulae (dentate in *M. barabanovi*) and shorted claws IV (*pt* of posterior primary branch of claws IV 37.9 – 49.9 in *M. huecoensis* **sp. nov**. vs. 51.7 – 66.7 in *M. barabanovi*)

Discussion

Tardigrade records from New Mexico are very scarce, as so far in only four studies records of tardigrades are provided (Beasley 1988; Mehlen 1969; Meyer & Hinton 2010; Pilato et al. 2007; records also reviewed in; Meyer 2013; Kaczmarek et al. 2016). Of the 21 species recorded, one of them (*Hypsibius macrocalcaratus* Beasley, 1988) has its type locality in this state, whereas two others have been described with material that included specimens from New Mexico (*Echiniscus viridianus* Pilato, Fontoura & Lisi, 2007 and *Milnesium zsalakoae* Meyer & Hinton, 2010). Regarding the genus *Mesobiotus*, only Beasley (1988) recorded it, making the present contribution the second record for this genus in New Mexico.

Claws elongation in tardigrades is hypothesized to be an adaptation to aquatic and glacial habitats, as longer claws help grip the substrate to avoid being dislodged by water currents; this trait has evolved multiple times convergently in tardigrades (Bertolani 1981; Zawierucha et al. 2018; Guidetti et al. 2019; Stec et al. 2022; Stec & Morek 2022). The phylogenetic position of this new species, completely unrelated to *M*. cf. *barabanovi* (another species with elongated claws), represents the first evidence of multiple independent evolution of elongated claws within a tardigrade genus. The claws of the new *Mesobiotus* species can be considered intermediate between standard *Mesobiotus* claws and

properly elongated ones (*pt* of posterior primary branch of claws IV in two typical *Mesobiotus* species: *M. diegoi* and *M. maklowiczi* do not exceed 36%, and it ranges from 37% and 50% in the new species, and between 51% and 67% in *M. barabanovi*). This intermediate claw elongation could hint to its adaptation to the peculiar habitat in which it has been found with periodic inundations followed by desiccation. However, a mechanicistic explanations on how exactly claw elongation relates to substrate type and inundation frequency are still elusive.

The sperm of the new species shows similarities with the two other *Mesobiotus* species for which sperm data are available, namely *Mesobiotus harmsworthi* (Murray 1907; Rebecchi et al. 2011) and *Mesobiotus* sp (Sugiura & Matsumoto 2021a). These new data on *Mesobiotus* sperm, together with the data on other Macrobiotidae genera sperm morphometry presented by Rebecchi (1997), Rebecchi et al. (2011) and Sugiura and Matsumoto (2021a, 2021b) allow us to hypothesize a genus level constant sperm size, with the model *Mesobiotus* sperm of intermediate size (range within *Mesobiotus* 45.6–52.7 μm, average values for $N = 3$ species) between *Macrobiotus* (range 18–38 μm, $N = 7$ species) and *Paramacrobiotus* (range 82.9–117 μm, $N = 3$ species); as noted by Sugiura and Matsumoto (2021a). *Mesobiotus* sperm is also easily differentiated from the other two considered Macrobiotidae genera sperm by the longer midpiece (*Mesobiotus* 4–11.5 μm, $N = 3$ species; *Macrobiotus* 2.3–4 μm, $N = 5$; *Paramacrobiotus* 3.4–4.5 μm, $N = 3$) (data on sperm morphometric from Guidi & Rebecchi 1996; Rebecchi 1997; Rebecchi & Guidi 1991; Rebecchi et al. 2000, 2011; Sugiura & Matsumoto 2021a, 2021c). This trend of uniformity in sperm morphometry within genera can be confirmed by examining additional species and will be helpful in providing more informative characters for delimitation of genera and overall taxonomy, as well as provide insight into the evolution of sperm in tardigrades. The mating behaviour also shows similarities with what described for another undescribed *Mesobiotus* species (Sugiura & Matsumoto 2021a). In particular multiple ejaculations at distance from the female seem to be a characteristic behavioral trait. The mounting behaviour also cannot be considered as an accidental cloaca contact, as the male curled up in the classic position observed in mounting males (Sugiura & Matsumoto 2021a).

Tardigrades from freshwater rock pools have been largely neglected. The very recent description of another species from this habitat type (*Acutuncus giovanniniae* Vecchi et al., 2023), together with the species described here, support the idea that these

ephemeral water basins could host a high biodiversity and endemism. The same patterns have been noted for rotifers in the same habitat (Schröder et al. 2007; Brown et al. 2020). The presence of endemic meiofauna (of which probably many more species are yet to be discovered) highlights the need for the study and preservation of this peculiar habitat and calls for efforts in overcoming the biodiversity knowledge shortfalls (Hortal et al. 2015) that prevent a comprehensive understanding of their biodiversity.

CRediT author statement

VM: Conceptualization, Methodology, Formal analysis, Data curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Funding acquisition. **MJL**: Conceptualization, Methodology, Writing - Original Draft, Writing - Review & Editing, Visualization. **CJ**: Methodology, Writing - Original Draft, Writing - Review & Editing, Visualization. **VT**: Methodology, Writing - Review & Editing. **WEJ**: Conceptualization, Resources, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition. **CS**: Resources, Writing - Review & Editing, Project administration, Funding acquisition.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Data availability statement

The data that support the findings of this study are openly available in Figshare at DOI:10.6084/m9.figshare.22656586 under CC-BY 4.0 license and in GenBank (see Table 1 for accession numbers).

Supplementary material

Supplemental data for this article can be accessed online at https://doi.org/10.1080/24750263.2023.2263033.

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