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Sisubiotus hakaiensis sp. nov. (Tardigrada, Macrobiotidae), a new tardigrade species from Calvert Island (British Columbia, Canada)

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Abstract. Tardigrades reports from British Columbia (Canada) trace back to 1908 and numerous species have been recorded from this region, despite the relatively few published sampling studies. We describe by integrative taxonomy (light microscopy morphology, morphometrics, and DNA sequencing) a new tardigrade species, *Sisubiotus hakaiensis* sp. nov. from the British Columbia central coast. The new species has been found in moss collected from a vertical rock outcrop near the Hakai Institute Calvert Island Field Station. *Sisubiotus hakaiensis* sp. nov. differs from all the other known species in the genus by the presence of a labyrinthine layer inside the egg process walls, whereas no consistent differences in the animals were found. This unique egg characteristic therefore required the amendment of the *Sisubiotus* generic diagnosis to account for the presence of the labyrinthine layer inside the egg process walls.

Keywords. Hakai, BC central coast, egg morphology, tardigrades, integrative taxonomy.

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Introduction

Reports of tardigrades from Canada can be traced back to two publications in the early 20th century: Richters (1908), who identified three species collected from Vancouver, British Columbia during a Pacific expedition from 1896–1897; and Murray (1910), who collected 31 species during his limited Canadian survey. To date, approximately 121 species of tardigrades are known in Canada, and almost half of these (58 species from 32 genera) were recorded from British Columbia (Meyer 2013; Kaczmarek

et al. 2016). Of 58 species known from British Columbia, six have their type locality in the province: *Echiniscus canadensis* Murray, 1910; *Echiniscus reymondi* Marcus, 1928; *Insuetifurca arrowsmithi* (Kathman & Nelson, 1989); *Ursulinius woodsae* (Kathman, 1990); *Macrobiotus occidentalis* Murray, 1910; and *Platicrista cheuleusis* Kathman, 1990. The genus *Sisubiotus* was recently erected (Stec *et al*. 2021a) based on both morphological and molecular data to accommodate for three species previously assigned to *Macrobiotus* C.A.S. Schultze, 1834. This genus can be distinguished from the otherwise similar *Macrobiotus* by the absence of cuticular pores, the presence of elongated teeth in the second band of the oral cavity armature (OCA), and by a characteristic egg morphology (laid free with areolation and conical processes without the labyrinthine layer) (Stec *et al*. 2021a). The first two described species, *S. spectabilis* (Thulin, 1928) and *S. grandis* (Richters, 1911), were considered for many years as species inquirenda (Dastych 1973). Maucci & Pilato (1974) showed that these two species are valid, and they can be discriminated by their morphology. *Sisubiotus wuyishanensis* (Zhang & Sun, 2014), described from China (Zhang & Sun 2014) was deemed to be insufficiently characterized to be differentiated from *S. spectabilis* and *S. grandis* and thus considered as species inquirenda by Stec *et al*. (2021a). Integrative taxonomy integrates multiple lines of evidence from multiple technique (morphological, molecular) to solve taxonomic issues, and it has been providing to be extremely useful in tardigrades taxonomy (see for example Kiosya *et al.* 2021; Stec *et al.* 2021b; Stec & Morek 2022). Thus, using an integrative taxonomy approach we here describe *Sisubiotus hakaiensis* sp. nov. from Calvert Island (British Columbia, Canada), which can be easily differentiated from the other three species in the genus by the presence of a labyrinthine layer in the egg processes. Therefore, we also provide an amended diagnosis of the genus considering this new character state for *Sisubiotus*.

Material and methods

Samples and specimens

Specimens were recovered from moss samples collected by one of the authors (HC) from a vertical rock outcrop near the Hakai Institute Calvert Island Field Station during a biodiversity survey in 2018 and on a subsequent collecting trip by Hakai researchers in 2021. The Field Station is located within the Hakai Lúxvbálís Conservancy of the Province of British Columbia on the central coast of British Columbia. The central coast of British Columbia extends from approximately the north end of Aristazabal Island at ~52°49′ N to the entrance to Queen Charlotte Strait at ~50°59′ N, excluding the offshore Haida Gwaii Archipelago (Lindstrom *et al*. 2021).

Details on samples and specimens found are shown in Table 1. The samples were examined for tardigrades using the protocol by Stec *et al*. (2015). Animals and eggs were split into several groups for specific analyses, i.e., morphological analysis in PCM (Phase Contrast Microscopy) and DNA sequencing (for details see Table 1).

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. Freshly mounted specimens were checked for the presence of sperm in the gonad (Coughlan & Stec 2019; Coughlan *et al.* 2019). Slides were examined under an Olympus BX53 light microscope with PCM, associated with an Olympus DP74 digital camera. All figures were assembled in Figure J (Mutteter & Zinck 2013). For structures that could not be satisfactorily focused on a single light microscope photograph, a stack of 2–3 images were taken with an equidistance of ca 0.2 µm and assembled manually into a single deep-focus image in GIMP ver. 2-10 (GIMP Development Team 2019). Deep-focus images obtained by stacking are indicated in the figures caption with an asterisk (*).

Table 1. Samples details and number of specimens analyzed [animals + eggs].

Sample and field numbers	Locality	Coordinates for all samples listed	Elevation m a.s.l.	Coll. date	Sample collector	Specimens analyzed [A+E]
S418 (HC2018-14-1)			50	15/06/2018	Henry Choong	PCM Paratypes [2+2]
S1910 (GSB-Loc.4)	Steep wall, trail to Lookout, Calvert Island	51°38′54″ N, 128°8′38″ W	40	29/06/2021	Gillian Sadlier-Brown	PCM Paratypes [0+1]
S1911 (GSB-Loc.5)			40	29/06/2021	Gillian Sadlier-Brown	PCM Holotype [1+0] + PCM Paratypes [52+0] + DNA [3+0]

Morphometrics and morphological nomenclature

All measurements are given in micrometers (µm). Sample size was adjusted following the recommendations by Stec *et al*. (2016). 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 OCA and eggshell morphology follow Kaczmarek & Michalczyk (2017). Morphometric data were handled using the "Parachela" ver. 1.7 template available from the Tardigrada Register (Michalczyk & Kaczmarek 2013). The raw morphometric data are provided as Supplementary file 1 (SM.01). Tardigrade taxonomy follows Bertolani *et al*. (2014) and Stec *et al*. (2021a).

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*. (2020a). We sequenced four DNA fragments, three nuclear (18S rRNA, 28S rRNA, ITS2) and one mitochondrial (COI). All fragments were amplified and sequenced according to the protocols described in Stec *et al*. (2020a); primers with original references are listed in Appendix 1. Sequencing products were read with an ABI 3130xl sequencer at the Department of Biological and Environmental Sciences (University of Jyväskylä, Finland).

Phylogenetic analysis

The phylogenetic analyses were conducted using concatenated 18S rRNA+28S rRNA+ITS-2+COI sequences. All *Sisubiotus* sequences available in GenBank were used. In addition, representative sequences of Macrobiotidae Thulin, 1928, Adorybiotidae Stec, Vecchi & Michalczyk, 2020, Richtersiusidae Guidetti, Schill, Giovannini, Massa, Goldoni, Ebel, Förschler, Rebecchi & Cesari, 2021 and Murrayidae Guidetti, Rebecchi & Bertolani, 2000 were included as outgroups (Appendix 2). Alignment of 18S and 28S markers was done on reference alignments as in Vecchi *et al*. (2022b). The ITS-2 sequences were aligned using MAFFT ver. 7 (Katoh *et al.* 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 (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' ver. 1.0.0 (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), partitions and models selection process and results are present in Supplementary file 2 (SM.02). Bayesian Inference (BI) phylogenetic reconstruction was done with MrBayes ver. 3.2.6 (Ronquist *et al.* 2012). Two runs with one cold chain and three heated chains were run for 20 million generations with a burning of 2 million generations, sampling a tree every 1000 generations. Posterior distribution sanity was checked with the Tracer ver. 1.7 (Rambaut *et al.* 2018). MrBayes input file with the input alignment is available as Supplementary file 3 (SM.03). The phylogenetic tree was visualized with FigTree ver. 1.4.4 (Rambaud 2007) and the image was edited with Inkscape 0.92.3 (Bah 2011). The complete phylogenetic tree is available in Supplementary file 4 (SM.04).

Results

Phylogenetic analysis

The phylogenetic analysis (Fig. 1) of the sequences from three individuals confirmed the generic assignation to *Sisubiotus* of the new species. The new species forms a separate clade in sister group

relationship with (*S. spectabilis*+*S. grandis*). The genera *Sisubiotus*, *Macrobiotus, Mesobiotus* Vecchi, Cesari, Bertolani, Jönsson, Rebecchi & Guidetti, 2016, *Tenuibiotus* Pilato & Lisi, 2011 and *Paramacrobiotus* Guidetti, Schill, Bertolani, Dandekar & Wolf, 2009 are recovered as monophyletic, whereas the genus *Minibiotus* is paraphyletic (SM.04).

Tardigrade taxa found

Other than the new species of *Sisubiotus*, the analysed samples contained:

Sample S418: *Adorybiotus* cf. *granulatus.*

Sample S1910: *Macrobiotus occidentalis* Murray, 1910.

Sample S1911: *Acanthechiniscus goedeni* (Grigarick, Mihelčič & Schuster, 1964), *Diploechiniscus* sp., *Hypechiniscus gladiator* (Murray, 1905), *Calohypsibius ornatus* (Richters, 1900), *Platicrista* sp., *Adorybiotus* cf. *granulatus.*

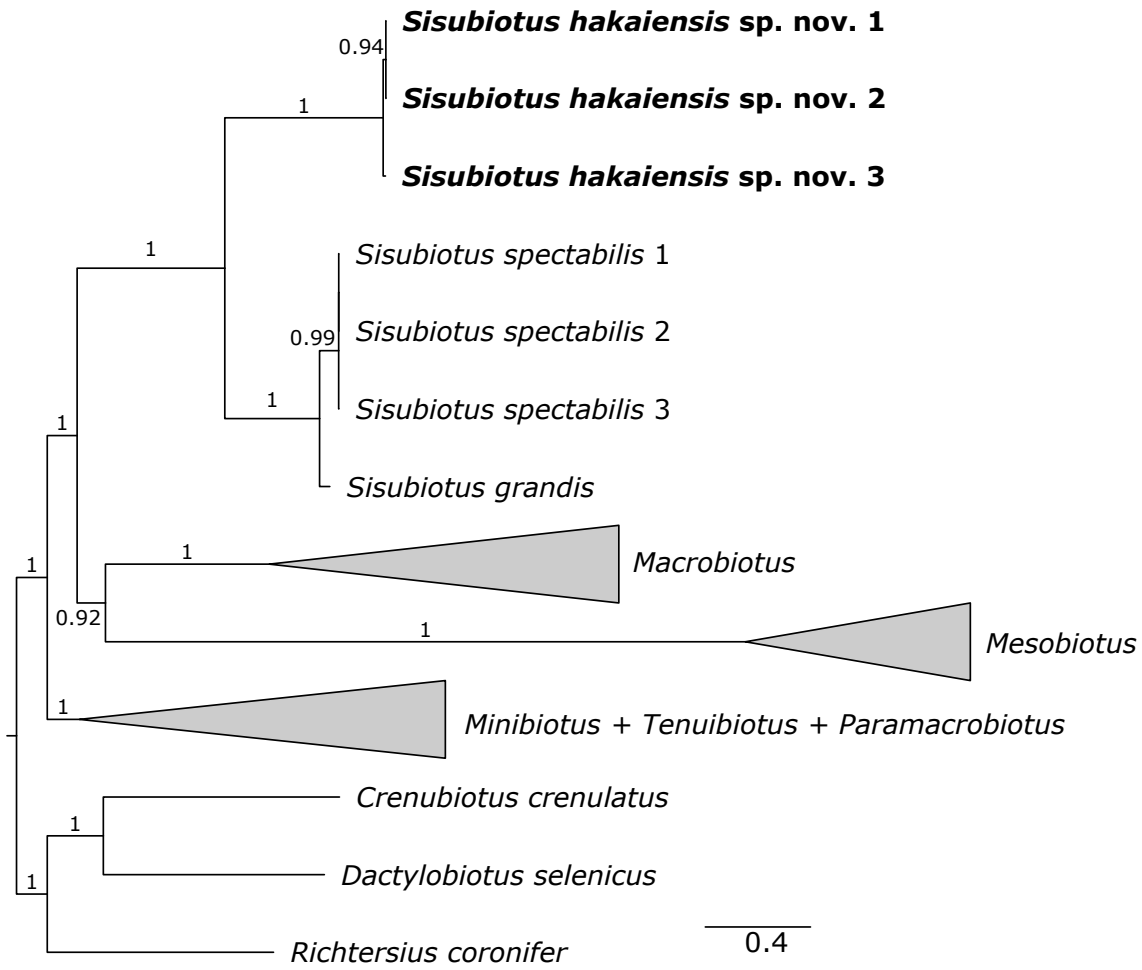

Fig. 1. Bayesian phylogenetic placement of the new species of *Sisubiotus* Stec, Vecchi, Calhim & Michalczyk, 2021. Values above/below branches are Bayesian posterior probability values. Scale bar indicates mutations/site.

Systematic and taxonomic account

Phylum Tardigrada Doyère, 1840
Class Eutardigrada Richters, 1926
Superfamily Macrobiotoidea Thulin, 1928
Family Macrobiotidae Thulin, 1928

Genus ***Sisubiotus*** Stec, Vecchi, Calhim & Michalczyk, 2021

Amended diagnosis

Large and whitish Macrobiotidae with: (i) a poreless cuticle, (ii) a wide rigid buccal tube with well-developed oral cavity armature (all three bands of teeth clearly visible in PCM, anterior teeth of the second band longitudinally elongated), (iii) two macroplacoids and a large microplacoid positioned close to them in the pharynx, (iv) Y-shaped claws of the Macrobiotus type with lunules on each leg, (v) ornamented eggs, laid freely, with areolation and conical processes with or without the labyrinthine layer.

Genus composition

Sisubiotus spectabilis (Thulin, 1928) (type species), *Sisubiotus grandis* (Richters, 1911), *Sisubiotus wuyishanensis* (Zhang & Sun, 2014) species inquirenda, *Sisubiotus hakaiensis* Vecchi, Choong & Calhim, 2022 sp. nov.

Sisubiotus hakaiensis sp. nov.
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Figs 2–5, Tables 2–3

Differential diagnosis

Reliable differences in differentiating the animal morphology between different species of *Sisubiotus* were not found. By the presence of a labyrinthine layer in the egg processes walls, *S. hakaiensis* sp. nov. can be easily differentiated from *S. spectabilis*, *S. grandis* and *S. wuyishanensis* (labyrinthine layer absent in these three species). In addition, *S. hakaiensis* differs from *S. wuyishanensis* by the presence of granulation on legs (present in *S. hakaiensis* vs absent in *S. wuyishanensis*) and by the shape of the egg processes walls (straight to slightly sigmoidal in *S. hakaiensis* vs concave in *S. wuyishanensis*).

Etymology

This species name refers to the Hakai Institute, which conducts and advances long-term scientific research at remote locations at the coastal margin of British Columbia, Canada, and which includes the Calvert Island Field Station from where the samples were collected.

Material examined

58 animals and 3 embryonated eggs. Specimens mounted on microscope slides in Hoyer's medium (55 animals + 3 embryonated eggs) and processed for DNA sequencing (3 animals).

Holotype

CANADA – **British Columbia** • Lookout, Calvert Island, British Columbia; 51°38′54″ N, 128°8′38″ W; 40 m a.s.l.; 29 Jun. 2021; Gillian Sadlier-Brown leg.; moss on rock; JYUt.S1911_SL5_B.

Paratypes

CANADA – **British Columbia** • 35 animals; Lookout, Calvert Island, British Columbia; 51°38′54″ N, 128°8′38″ W, 40–50 m a.s.l.; 15 Jun. 2018, 29 Jun. 2021; Henry Choong & Gillian Sadlier-Brown leg.; moss on rock; JYVt.S418_SL2, JYVt.S1911_SL1 to SL6 • 19 animals; same collection data as for

preceding; RBCM.S1911_SL9, SL_10 (RBCM 022-00001-002) • 2 embryonated eggs; same collection data as for preceding; JYVt.S418_SL1 • 1 embrionated egg; same collection data as for preceding; RBCM.S1910_SL2_A (RBCM 022-00001-001).

Voucher specimens are deposited in the Natural history collections of the Jyväskylä University Museum, Ihantolantie 5, Jyväskylä, Finland (JYV), Survontie 9, 40520 Jyväskylä, Finland (Slides JYVt.S1911_SL5, JYVt.S418_SL1-2, JYVt.S1911_SL1 to SL6) and in the Invertebrate Zoology Department, Royal BC Museum (RBCM), 675 Belleville Street, Victoria, BC, Canada (RBCM.S1910_SL2 (RBCM 022-00001-001), RBCM.S1911_SL9, SL10 (RBCM 022-00001-002)) .

Description

Animals (measurements and statistics in Table 2)

Body whitish; after fixation in Hoyer's medium body transparent (Fig. 2A). Eyes present in animals before and after fixation in Hoyer's medium. Cuticle poreless. Patches of fine granulation on the internal and external surfaces of legs I–III (Fig. 2B–C) as well as dorsal and dorso lateral of legs IV clearly visible in PCM (Fig. 2D). A pulvinus is present on the internal surface of legs I–III (Fig. 2B). Claws slender, of the *hufelandi* type. Primary branches with distinct accessory points, a long common tract, and with an evident stalk connecting the claw to the lunula (Fig. 3). All lunulae smooth (Fig. 3A, D). Single cuticular bar on legs I–III often visible in PCM (Fig. 3C), whereas the horseshoe-shaped structure under

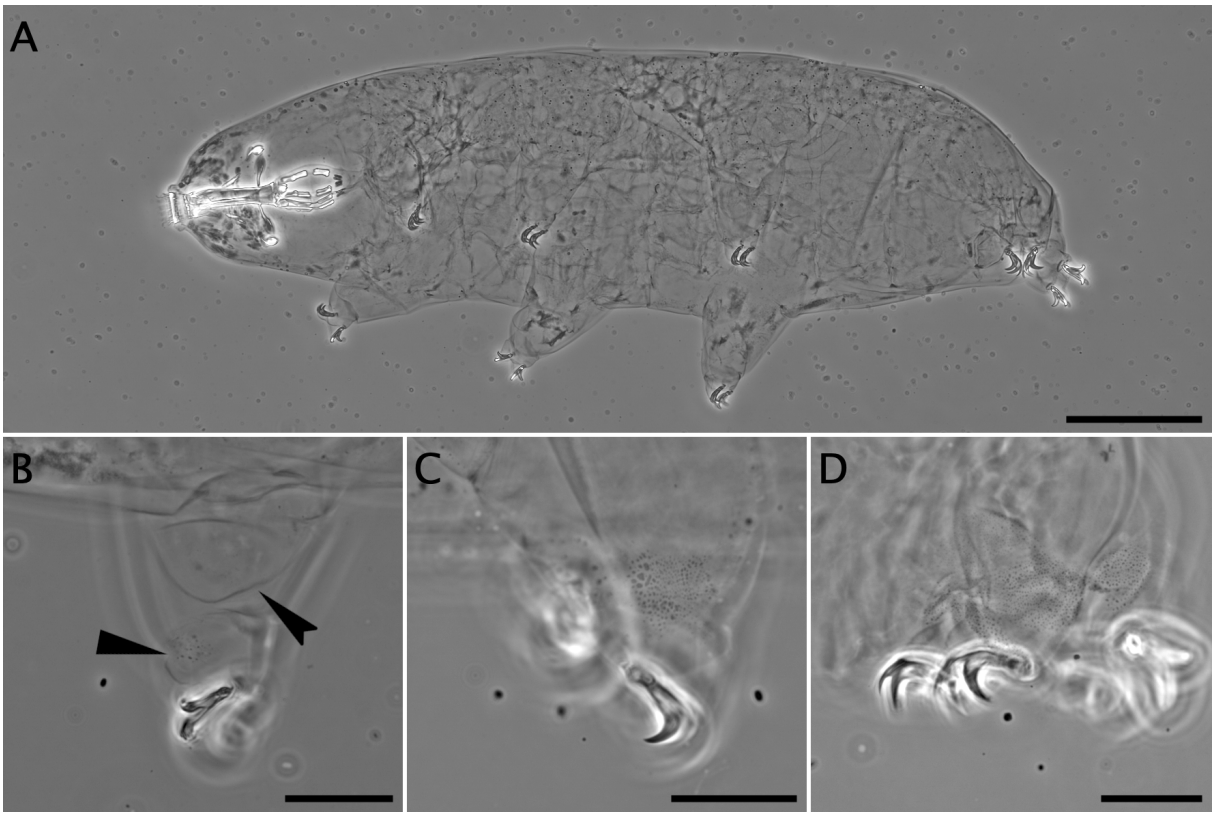

Fig. 2. *Sisubiotus hakaiensis* sp. nov., habitus in PCM. **A**. Holotype (JYUt.S1911_SL5_B, Hoyer's medium), dorso-ventral projection. **B***. Paratype (JYUt.S1911_SL3_A). Pulvinus (indented arrowhead) and granulation (arrowhead) on the internal side of leg I. **C–D**. Paratype (JYUt.S1911_SL4_C). **C**. Granulation on external side of leg II. **D**. Granulation on claws IV. Deep-focus images obtained by stacking are indicated in the figures caption with an asterisk (*). Scale bars: A = 100 µm; B–D = 20 µm.

Table 2. Measurements (in µm) of selected morphological structures of the animals of *Sisubiotus hakaiensis* sp. nov. mounted in Hoyer's medium. Abbreviations: N = number of eggs/structures measured; Range = the smallest and the largest structure among all measured specimens; SD = standard deviation.

Character	N	Range		Mean		SD		Holotype	
		µm	*pt*	µm	*pt*	µm	*pt*	µm	*pt*
Body length	25	387–685	*707–1070*	573	*948*	72	*87*	650	*1034*
Buccopharyngeal tube									
Buccal tube length	30	35.7–73.3	–	58.3	–	8.2	–	62.9	–
Stylet support insertion point	30	30.1–61.7	*78.9–85.4*	48.5	*83.2*	7.1	*1.4*	52.6	*83.6*
Buccal tube external width	25	5.7–14.4	*15.2–21.2*	10.6	*18.4*	2.1	*1.4*	11.9	*19.0*
Buccal tube internal width	25	3.7–11.5	*10.3–17.7*	8.3	*14.4*	1.7	*1.5*	10.0	*15.8*
Ventral lamina length	25	25.5–51.7	*65.2–75.1*	40.9	*70.7*	6.2	*2.6*	42.2	*67.1*
Placoid lengths									
Macroplacoid 1	30	7.1–21.0	*17.7–36.4*	16.0	*27.3*	3.5	*4.0*	17.7	*28.1*
Macroplacoid 2	30	4.5–15.5	*12.7–21.2*	10.2	*17.3*	2.2	*1.9*	11.6	*18.4*
Microplacoid	30	3.1–7.6	*7.3–11.4*	5.5	*9.4*	1.0	*1.0*	5.7	*9.1*
Macroplacoid row	30	13.5–37.0	*34.6–57.2*	29.0	*49.3*	6.0	*5.4*	32.1	*51.0*
Placoid row	30	17.9–46.1	*46.0–72.5*	36.9	*62.7*	7.2	*6.1*	41.8	*66.4*
Claw 1 heights									
External primary branch	19	7.4–15.5	*19.4–23.2*	12.8	*21.5*	1.7	*1.0*	13.7	*21.8*
External secondary branch	19	5.4–12.0	*14.9–18.0*	10.0	*16.8*	1.4	*0.9*	10.3	*16.4*
Internal primary branch	18	9.6–13.7	*17.1–21.9*	12.3	*20.3*	1.1	*1.3*	13.7	*21.8*
Internal secondary branch	17	8.4–10.9	*12.6–17.6*	9.8	*16.1*	0.7	*1.3*	10.9	*17.4*
Claw 2 heights									
External primary branch	22	6.9–14.5	*19.2–24.2*	12.9	*21.5*	1.6	*1.6*	14.2	*22.5*
External secondary branch	22	4.5–12.2	*12.5–18.9*	9.8	*16.3*	1.6	*1.6*	11.5	*18.3*
Internal primary branch	22	5.6–14.4	*15.6–23.0*	12.2	*20.3*	1.8	*1.9*	14.4	*22.9*
Internal secondary branch	20	8.8–11.3	*14.5–17.9*	9.9	*16.4*	0.7	*1.0*	11.0	*17.4*
Claw 3 heights									
External primary branch	18	8.1–15.2	*18.9–23.9*	13.0	*22.0*	1.6	*1.2*	14.2	*22.6*
External secondary branch	16	6.7–12.6	*15.5–18.7*	10.3	*17.5*	1.4	*1.0*	11.3	*17.9*
Internal primary branch	19	7.6–14.6	*17.8–23.1*	12.6	*21.3*	1.6	*1.5*	14.5	*23.0*
Internal secondary branch	18	5.3–12.0	*13.5–18.8*	9.9	*16.9*	1.4	*1.4*	11.6	*18.4*
Claw 4 heights									
Anterior primary branch	19	9.8–20.1	*22.0–37.3*	17.1	*29.6*	2.4	*3.5*	20.1	*31.9*
Anterior secondary branch	19	6.3–14.5	*15.7–26.8*	12.2	*20.8*	1.9	*2.7*	14.5	*23.1*
Posterior primary branch	20	8.8–23.6	*21.5–43.1*	16.1	*27.7*	2.8	*4.7*	19.4	*30.8*
Posterior secondary branch	18	6.1–13.8	*14.6–23.0*	11.3	*19.3*	1.8	*2.4*	13.4	*21.3*

claws IV poorly visible only in PCM (Fig. 3D). Mouth antero-ventral. Bucco-pharyngeal apparatus of the *Macrobiotus* type (Fig. 4A), with the ventral lamina and ten peribuccal lamellae. Pharyngeal bulb spherical, with trapezoidal apophyses with a median constriction, two rod-shaped macroplacoids and a large microplacoid positioned close to them; Fig. 4B–C). The macroplacoid length sequence is 2 < 1. The first macroplacoid is anteriorly narrowed and constricted in the middle whereas the second has a sub-terminal constriction (Fig. 4B–C). The oral cavity armature well developed and composed of three bands of teeth, always clearly visible under PCM (Fig. 4D–E). The first band of teeth is composed of numerous small teeth visible in PCM as granules (Fig. 4D–E), arranged in several rows, situated anteriorly in the oral cavity, which start behind the bases of the peribuccal lamellae and extend on the lamellae bases. The second band of teeth is situated between the ring fold and the third band of teeth and comprised of 3–4 rows of teeth visible in PCM as granules (Fig. 4D–E) larger than those in the first band. The most anterior row of teeth within the second band comprises larger and longitudinally elongated teeth than the subsequent posterior rows (Fig. 4D–E). The teeth of the third band are located within the posterior portion of the oral cavity, between the second band of teeth and the buccal tube anterior ending (Fig. 4D–E). The third band of teeth is divided into the dorsal and the ventral portion. Under PCM, both bands are divided into three distinct transverse ridges, with the medio-dorsal larger than the medio-ventral one. In some specimens, additional mucrones can occur behind the medio-ventral ridge (Fig. 4E). Typically-shaped stylet furca, with spherical condyles supported by short branches provided with small apophyses (Fig. 4A).

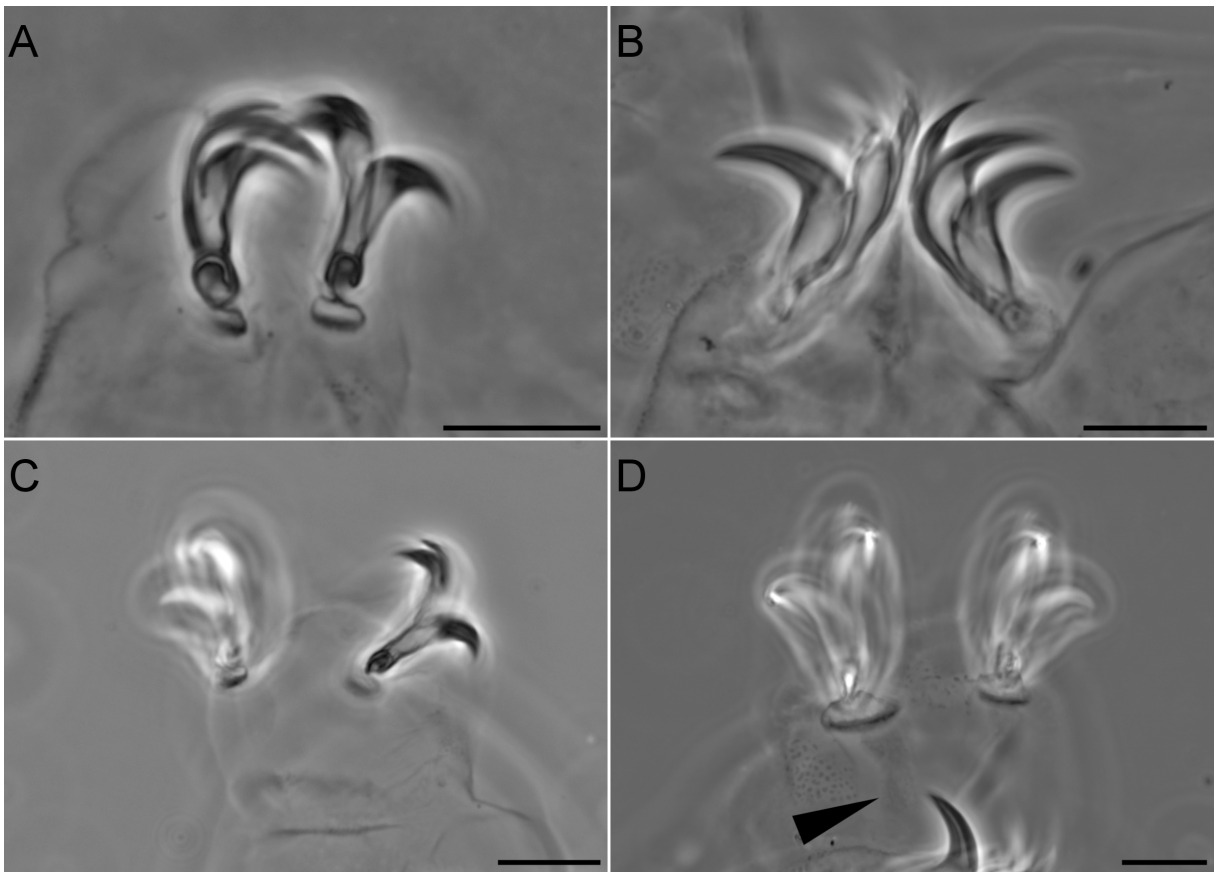

Fig. 3. *Sisubiotus hakaiensis* sp. nov., claws in PCM. **A*, C**. Paratype (JYUt.S1911_SL3_A). Claws II–III. **B, D**. Holotype (JYUt.S1911_SL5_B). Claws IV. Arrowhead indicates horseshoe-shaped structure under claws IV. Deep-focus images obtained by stacking are indicated in the figures caption with an asterisk (*). Scale bars =10 μm.

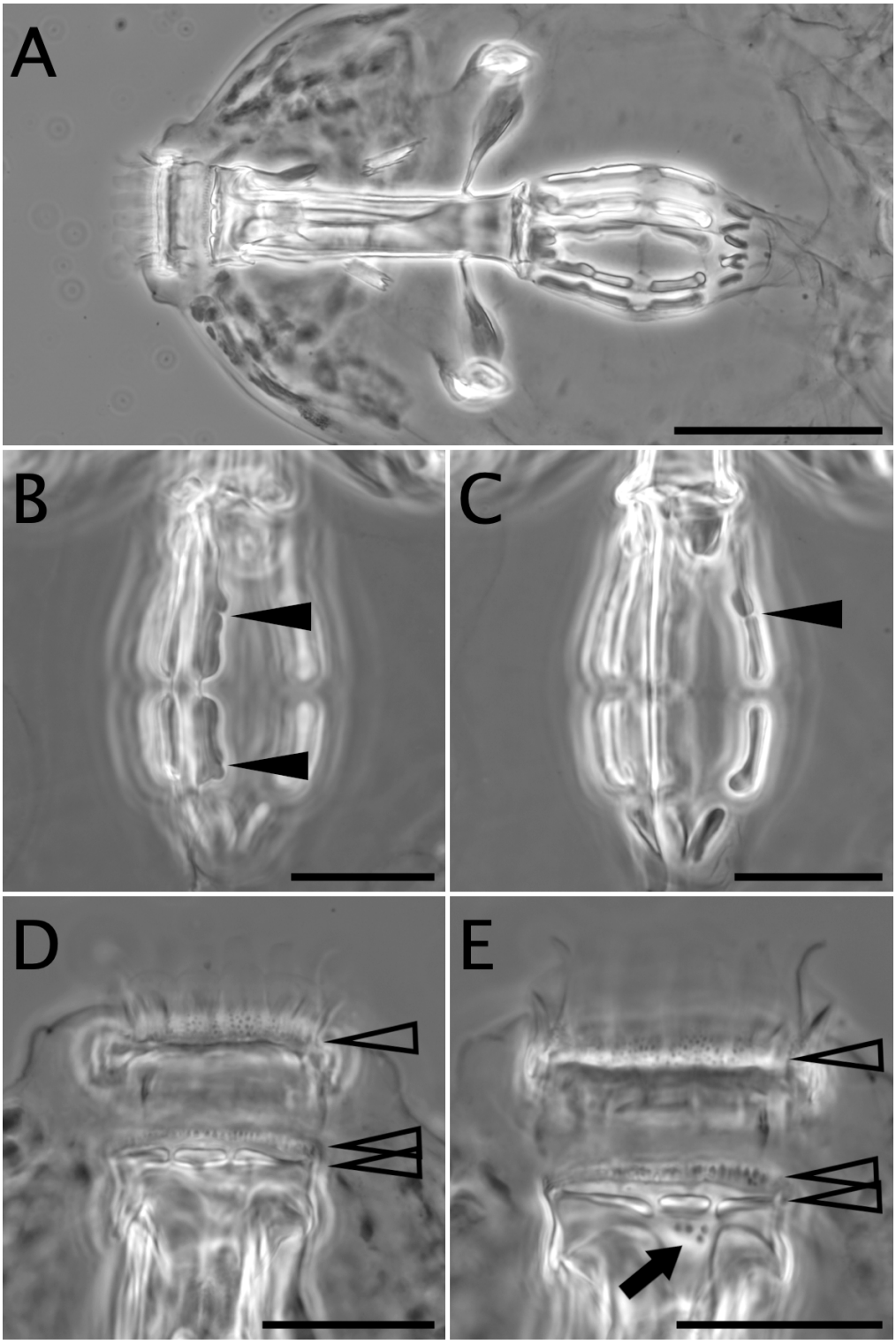

Fig. 4. *Sisubiotus hakaiensis* sp. nov., buccal-pharyngeal apparatus in PCM. **A, D–E**. Paratype (JYUt. S1911_SL4_C). **B–C**. Paratype (JYUt.S1911_SL2_B). **A**. In toto buccal-pharyngeal apparatus. **B–C**. Placoids, arrowheads indicate constrictions in the macroplacoids. **D***. Dorsal Oral Cavity Armature (OCA), empty arrowheads indicate the three bands of the OCA. **E***. OCA, empty arrowheads indicate the three bands of the OCA. Deep-focus images obtained by stacking are indicated in the figures caption with an asterisk (*). Scale bars: A = 50 µm; B–E = 20 µm.

Eggs (measurements and statistics in Table 3)
Laid freely, white, spherical with large conical processes, areolated (Fig. 5A). About ten processes on the circumference. Each process is surrounded by usually eight to twelve deep areolae. Usually, two rows of areolae are present between the neighboring processes (Fig. 5A). The areolae rims are thin and high, and the areolae surface is reticulated (Fig. 5B). The labyrinthine layer between the process walls present and composed by a very fine mesh (Fig. 5C–D). Processes walls straight to slightly sigmoidal, and processes tips usually blunted or flat (Fig. 5C–D).

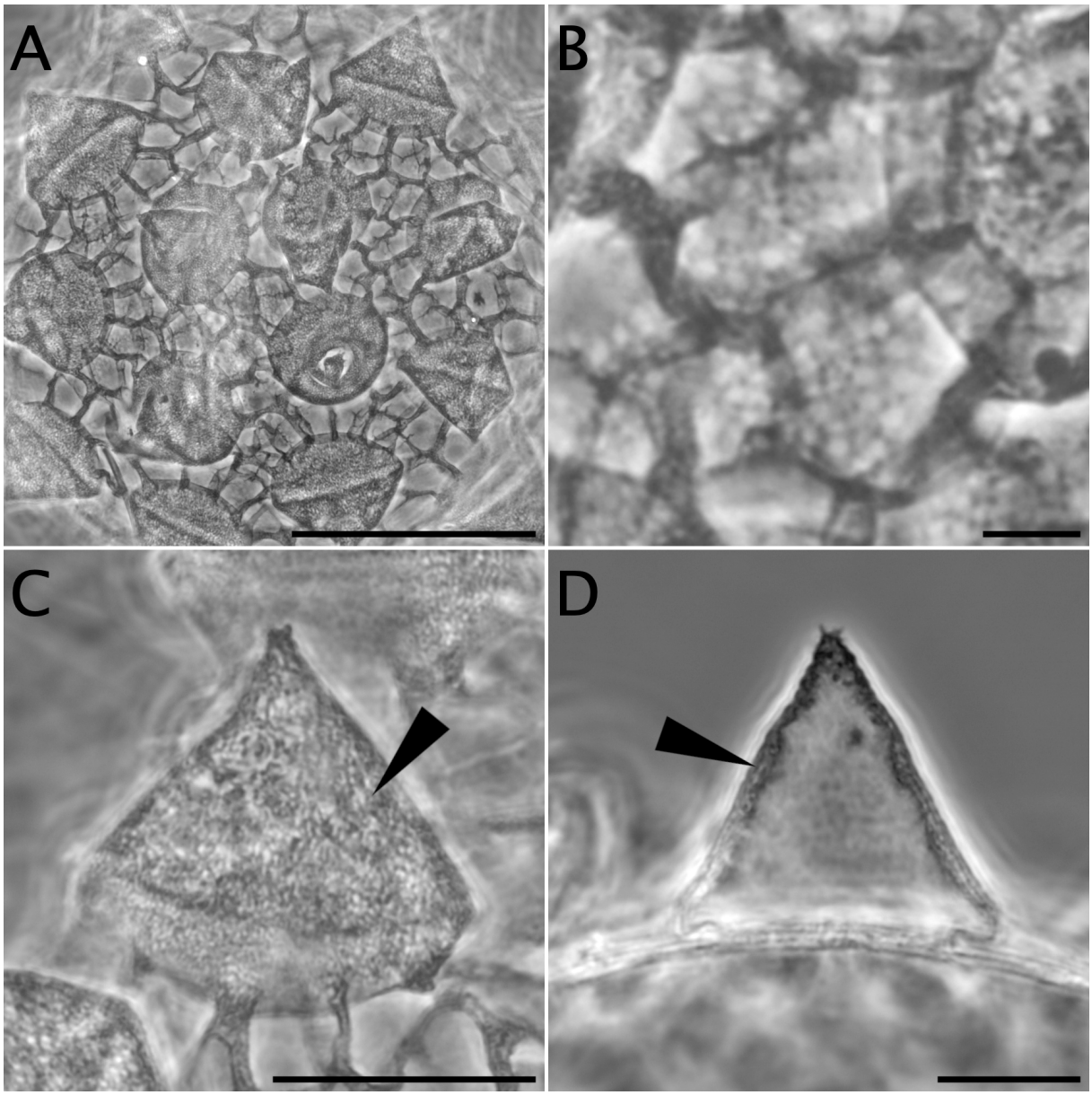

Fig. 5. *Sisubiotus hakaiensis* sp. nov. Paratype (JYUt.S418_SL1_A). Eggs in PCM. **A**. Egg surface. **B**. Reticulated chorion between areolations. **C**. Egg process with labyrinthine layer (arrowhead); seen as reticulation. **D**. Egg process in section showing labyrinthine layer (arrowhead). Scale bars: A = 50 µm; B = 5 µm; C = 20 µm; D = 10 µm.

Table 3. Measurements (in µm) of selected morphological structures of the eggs of *Sisubiotus hakaiensis* sp. nov. mounted in Hoyer's medium. Abbreviations: N = number of eggs/structures measured; Range = the smallest and the largest structure among all measured specimens; SD = standard deviation.

Character	N	Range	Mean	SD
Egg bare diameter	2	126.0–134.0	130.0	5.7
Egg full diameter	1		171.0	
Process height	9	15.6–31.4	25.8	5.2
Process base width	9	18.6–29.2	23.9	3.0
Process base/height ratio	9	71%–170%	98%	32%
Inter-process distance	6	3.2–11.7	7.5	3.7
Number of processes on the egg circumference	1		10.0	

Reproduction

The examination of animals freshly mounted in Hoyer's medium revealed the presence of testis filled with sperm, so this species can be considered gonochoric.

DNA sequences

Sequences from 3 individuals from sample S1911 were obtained. 18S rRNA (3 sequences: OM523054-6); 28S rRNA (3 sequences: OM523059-61); COI (2 sequences: OM523181-2); ITS2 (2 sequences: OM523057-8).

Discussion

The analyzed animals and eggs belong to a new species in the genus *Sisubiotus*, morphologically and phylogenetically distinct from all other species in the genus. The finding of three embryonated eggs allowed us to link the animal and eggs morphologies. The presence of a labyrinthine layer in the egg processes walls is a new character state for the genus, requiring an amendment of its morphological diagnosis. This newly described species increases the number of tardigrade species recorded from British Columbia to 59, of which seven have their type locality specifically in this province. This new species finding highlights how important is to keep sampling in already explored areas as more biodiversity still awaits to be found (see for example Vuori *et al.* 2020).

The genus *Sisubiotus* has been reported mostly from Europe (Croatia, Finland, France, Italy, Norway, Poland, Romania, Switzerland), the Arctic (Franz Josef Land) and Asia (China, Japan, Siberia-Russia) (McInnes 1994; Stec *et al.* 2021a). Only a few records are known for the Americas: *S. grandis* was found in Greenland and Alaska (Grøngaard *et al.* 1990; Johansson *et al.* 2013) whereas *S. spectabilis* was recorded for Greenland (Maucci 1996), Alaska (USA) (Dastych 1982; Johansson *et al*. 2013), West Virginia (USA) (Tarter & Nelson 1993) and Argentina (Claps & Rossi 1984). The current description of *S. hakaiensis* sp. nov. is a valuable addition to the scarce records of this genus in the Americas. It is important to note that reticulation in the egg process is a unique feature in an already uncommon and peculiar genus. Thus, it is unlikely to have been overlooked in previous records of *Sisubiotus* from the Americas. Nevertheless, doubts remain with respect to the records of *S. grandis* and *S. spectabilis*: the

considerable distance to their respective type localities and geographical distribution could be indicative of separate (and likely cryptic/pseudocryptic) species being present in the Americas.

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Supplementary files

Supp. file 1. SM.01. Morphometric data for *Sisubiotus hakaiensis* sp. nov.
https://doi.org/10.5852/ejt.2022.823.1815.6991

Supp. file 2. SM.02. Model selection results on the concatenated alignment.
https://doi.org/10.5852/ejt.2022.823.1815.6993

Supp. file 3. SM.03. MrBayes input file for phylogenetic analysis including the used alignment.
https://doi.org/10.5852/ejt.2022.823.1815.6995

Supp. file 4. SM.04. Output phylogenetic tree from MrBayes analysis.
https://doi.org/10.5852/ejt.2022.823.1815.6997

Appendix 1. PCR primers for amplification of the four DNA fragments sequenced in the study.

DNA fragment	Primer name	Primer direction	Primer sequence (5′–3′)	Primer source
18S rRNA	18S_Tar_1Ff	Forward	AGGCGAAACCGCGAATGGCTC	Stec *et al*. (2017)
	18S_Tar_1Rr	Reverse	GCCGCAGGCTCCACTCCTGG	
28S rRNA	28S_Eutar_F	Forward	ACCCGCTGAACTTAAGCATAT	Gąsiorek *et al*. (2018)
	28SR0990	Reverse	CCTTGGTCCGTGTTTCAAGAC	Mironov *et al*. (2012)
ITS-2	Eutar_Ff	Forward	CGTAACGTGAATTGCAGGAC	Stec *et al*. (2018a)
	Eutar_Rr	Reverse	TCCTCCGCTTATTGATATGC	
COI	LCO1490	Forward	GGTCAACAAATCATAAAGATATTGG	Folmer *et al*. (1994)
	HCO2198	Reverse	TAAACTTCAGGGTGACCAAAAAATCA	

Appendix 2. GenBank accession numbers of DNA sequences used in the phylogenetic analysis.

	18S	28S	COI	ITS2	References
Sisubiotus hakaiensis sp. nov. 1	OM523054	OM523059	OM523181	OM523057	This study
Sisubiotus hakaiensis sp. nov. 2	OM523055	OM523060	OM523182	OM523058	This study
Sisubiotus hakaiensis sp. nov. 3	OM523056	OM523061			This study
Sisubiotus spectabilis 1	MN888371	MN888357	MN888323	MN888331	Stec *et al.* 2021a
Sisubiotus spectabilis 2	MN888371	MN888357	MN888322	MN888331	Stec *et al.* 2021a
Sisubiotus spectabilis 3	MN888372	MN888364	MN888324	MN888344	Stec *et al.* 2021a
Sisubiotus grandis		MH079490			Guil *et al.* 2019
Crenubiotus crenulatus	MT812474	MT812468	MT808079	MT812606	Stec *et al.* 2020b
Dactylobiotus selenicus	MT812476	MT812466	MT808076	MT812602	Stec *et al.* 2020b
Macrobiotus ariekammensis	MZ463668	MZ463674	MZ460999	MZ463656	Stec *et al.* 2022
Macrobiotus pallarii	MT809069	MT809081	MT807924	MT809094	Stec *et al.* 2021b
Macrobiotus polypiformis	KX810008	KX810009	KX810011	KX810010	Roszkowska *et al.* 2017
Mesobiotus harmsworthi	MH197146	MH197264	MH195150	MH197154	Kaczmarek *et al.* 2018
Mesobiotus insanis	MF441488	MF441489	MF441491	MF441490	Mapalo *et al.* 2017
Mesobiotus philippinicus	KX129793	KX129794	KX129796	KX129795	Mapalo *et al.* 2016
Minibiotus cf. *diversus*	OK663227	OK663238	MW306859	OK663216	Vecchi *et al.* 2022a, 2022b
Minibiotus ioculator	MT023998	MT024041	MT023412	MT024000	Stec *et al.* 2020a
Minibiotus pentannulatus	MT023999	MT024042	MT023413	MT024001	Stec *et al.* 2020a
Paramacrobiotus areolatus	MH664931	MH664948	MH675998	MH666080	Stec *et al.* 2020c
Paramacrobiotus fairbanksi	MH664941	MH664950	MH676011	MH666090	Stec *et al.* 2020c
Paramacrobiotus lachowskae	MF568532	MF568533	MF568534	MF568535	Stec *et al.* 2018b
Paramacrobiotus richtersi	OK663224	OK663236	OK662995	OK663213	Vecchi *et al.* 2022b
Paramacrobiotus tonollii	MH664946	MH664963	MH676018	MH666096	Stec *et al.* 2020c
Richtersius coronifer	MH681760	MH681757	MH676053	MH681763	Stec *et al.* 2020d
Tenuibiotus cf. *ciprianoi*	MN888376	MN888361	MN888328	MN888348	Stec *et al.* 2021a
Tenuibiotus danilovi	MN888377	MN888362	MN888329	MN888349	Stec *et al.* 2021a
Tenuibiotus voronkovi	KX810045	KX810049	KX810042	KX810046	Zawierucha *et al.* 2016