

## Article

# Integrative Descriptions of Two New Tardigrade Species along with the New Record of *Mesobiotus skorackii* Kaczmarek et al., 2018 from Canada

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**Abstract:** Two new tardigrade species from a moss sample collected in Canada, one representing *Macrobiotus hufelandi* complex and the second one belonging to the genus *Bryodelphax*, are described. Integrative analysis was undertaken based on morphological and morphometric data (using both light and scanning electron microscopy (SEM)) combined with multilocus molecular analysis (nuclear sequences, i.e., 18S rRNA, 28S rRNA and ITS-2 as well as mitochondrial COI barcode sequences). Based on COI sequences, *Macrobiotus birendrai* sp. nov. is most similar to *Mac. canaricus* (p-distance 17%), whereas *Bryodelphax mareki* sp. nov. is most similar to *Bry. parvulus* (p-distance 16%). Both species differ also from their congeners in some morphological and morphometric characters of adults and/or details of egg chorion. Additionally, a large population of *Mesobiotus skorackii* was found in the sample and this is the first report of this species outside its terra typica in Kirghizia. The original description of this species was prepared based solely on the morphology and morphometry, therefore, here we provide updated data for this species enclosing morphometric and molecular data for the Canadian population.

**Keywords:** *Bryodelphax mareki* sp. nov.; DNA barcoding; Eutardigrada; Heterotardigrada; *Macrobiotus birendrai* sp. nov.; water bears



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## 1. Introduction

Canada is the second largest country in the world which extends its longitude from approximately 52° to 141° W to latitude approximately 42° to 83° N. It has such a distance that spans in six time zones and has a wide variety of climates. The highest peak in Canada which is Mount Logan reaches 5959 m asl and the country's landform structure can be considered a vast basin. Additionally, people living in two-thirds of the area experience very cold winters and short, cool summers. However, the interior plains of central southern area come with very cold winters, hot summers, and relatively sparse precipitation. Nonetheless, climate with hot, humid summers and cold, snowy winters also prevails in Southern Ontario and Quebec. Except for the west coast, all of Canada has a

winter season with average temperatures below freezing and with continuous snow cover (<https://www.britannica.com/place/Canada> (accessed on 18 June 2021)).

Tardigrada, also commonly known as water bears, inhabit in terrestrial and aquatic (freshwater and marine) environments. They can be found on aquatic plants and/or in lichens, leaf litter, mosses, soil, sediments [1–3]. To date, more than ca. 1300 species of tardigrades have been described throughout the world [4–7]. The genus *Bryodelphax* [8] is unique amongst Echiniscidae with some peculiar apomorphies like presence of 10 peribuccal papulae and plesiomorphies like ancestral type of the buccal apparatus, which makes *Bryodelphax* a good example of mosaic evolution in tardigrades [9,10]. Moreover, it is characterized by the presence of median plates 1 and 2 divided, median plate 3 not divided, and absence of notches on terminal plate. Up to now, 26 species were attributed to this genus [7]. The genus *Macrobiotus* [11] is one of the most species-rich and widespread genus in the phylum being also, the first formally described tardigrade genus. It is characterized by the presence of a rigid buccal tube with a straight ventral lamina lacking a ventral hook, 10 peribuccal lamellae, pharynx with two macropylacoids and micropylacoid, symmetrical diploclaws and freely laid ornamented eggs [12]. Up to now, 118 species were attributed to this genus [7].

Tardigrade fauna of Canada is rather poorly known and up to now only 121 species have been reported from this region [13,14]. In this study, we applied integrative taxonomy for description of two new species from Canada belonging to the genus *Bryodelphax* and the *Macrobiotus hufelandi* complex. Moreover, we enriched this paper in additional molecular and morphometric data of the Canadian record of *Mesobiotus skorackii* Kaczmarek, Zawierucha, Buda, Stec, Gawlak, Michalczyk and Roszkowska [15], as the original description of this species was prepared based solely on the morphology and morphometry.

## 2. Materials and Methods

### 2.1. Sampling

A moss sample was collected in Banff National Park (AB, Canada) in March 2019. It was then packed in a paper envelope, dried at a temperature of ca. 20 °C and delivered to the Department of Animal Taxonomy and Ecology at the Faculty of Biology, Adam Mickiewicz University in Poznań (Poland). The tardigrade collection, extraction and mounting techniques followed the protocol of Stec et al. [16].

### 2.2. Microscopy and Imaging

In total, 163 animals (74 *Bryodelphax mareki* sp. nov. + 45 *Macrobiotus birendrai* sp. nov. + 44 *Mesobiotus skorackii*) and 31 eggs (12 *Macrobiotus birendrai* sp. nov. + 19 *Mesobiotus skorackii*) were mounted on microscope slides in the Hoyer's medium, and then examined under Olympus BX41 Phase Contrast light Microscope (PCM) associated with Olympus SC50 digital camera (Olympus Corporation, Shinjuku-ku, Japan). The 44 animals and 8 eggs were prepared for scanning electron microscopy (SEM) analysis according to the protocol in Roszkowska et al. [17] and examined under high vacuum in Hitachi S3000N SEM. Thirty-one specimens were prepared for genotyping.

All figures were assembled in Corel Photo-Paint 2017. For deep structures that could not be fully focused in a single photograph, a series of 2–10 images were taken every ca. 0.5 µm and then manually assembled into a single deep-focus image in Corel Photo-Paint 2017.

### 2.3. 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 end of the body, excluding hind legs. The *sp* index in *Bryodelphax* is the ratio of the length of a given structure to the length of the scapular plate expressed as a percentage (length of structure × 100/length scapular plate) [18] and later independently proposed as the *psc* index by Fontoura and Morais [19]. Ventral plates configuration in *Bryodelphax* is given

according to Kaczmarek et al. [20]. The types of bucco-pharyngeal apparatuses and claws of Macrobiotidae were classified according to Pilato and Binda [21]. All measurements and terminology of adults and eggs of Macrobiotidae were prepared according to Kaczmarek and Michalczyk [22] and Kaczmarek et al. [23]. Terminology describing the oral cavity armature (OCA) in *Macrobiotus* and *Mesobiotus* follows Michalczyk and Kaczmarek [24] and OCA morphotypes are given according to Kaczmarek and Michalczyk [22]. The macroplacoid length sequence in *Macrobiotus* and *Mesobiotus* was indicated according to Kaczmarek et al. [25]. The *pt* ratio is the ratio of the length of a given structure to the length of the buccal tube expressed as a percentage [26]. The terminology of cuticular bars in macrobiotid legs follows Kiosya et al. [27]. The classification of type of egg process, sculpture on egg processes, egg processes bases and egg shell surface between processes are given according to Kaczmarek et al. [23]. Genus abbreviations follow Perry et al. [28].

Morphometric data were handled using the “Parachela” ver. 1.8 and “Echiniscoidea” ver. 1.4 template available from the Tardigrada Register [29].

#### 2.4. Comparative Material

For identification and differentiation of the new species, the key by Gąsiorek et al. [30] for the genus *Bryodelphax* and the key by Kaczmarek and Michalczyk [22] for the genus *Macrobiotus* were used. We also compared our new species with the type material of *Bry. aaseae* Kristensen, Michalczyk and Kaczmarek [9], *Bry. asiaticus* Kaczmarek and Michalczyk [31], *Bry. brevidentatus* Kaczmarek, Michalczyk and Degma [32], *Bry. olshanzowskii* Kaczmarek, Parnikoza, Gawlak, Esefeld, Peter, Kozeretska and Roszkowska [33], *Bry. parvuspolaris* Kaczmarek, Zawierucha, Smykla and Michalczyk [20], *Mac. dulcipuris* Roszkowska, Gawlak, Draga and Kaczmarek [34], *Mac. kazmierskii* Kaczmarek and Michalczyk, [35], *Mac. marlenae* Kaczmarek and Michalczyk [36], *Mac. paulinae* Stec, Smolak, Kaczmarek and Michalczyk [16], *Mac. pisacensis* Kaczmarek, Cytan, Zawierucha, Diduszko and Michalczyk [25], *Mac. polonicus* Pilato, Kaczmarek, Michalczyk and Lisi [37], *Mac. polypiformis* Roszkowska, Ostrowska, Stec, Janko and Kaczmarek [38], *Mac. porifini* Kuzdrowska, Mioduchowska, Gawlak, Bartylak, Kepel, Kepel and Kaczmarek [39], *Mac. sottilei* Pilato, Kiosya, Lisi and Sabella [40] and *Mac. wandae* Kayastha, Berdi, Miaduchowska, Gawlak, Łukasiewicz, Gołdyn and Kaczmarek [41].

#### 2.5. Genotyping

Prior to DNA extraction, individual tardigrades from the three species were preliminarily identified *in vivo* using light microscopy (LM). Genomic DNA was extracted using a Chelex<sup>®</sup> 100 resin (Bio-Rad, Hercules, CA, USA) extraction method [42] with modification in order to obtain voucher specimens, i.e., tardigrade exoskeletons [43]. After DNA extraction we performed morphological analysis following the protocol of Kaczmarek et al. [43]. Then, all exoskeletons were deposited in the collection of the Department of Animal Taxonomy and Ecology, Faculty of Biology, Adam Mickiewicz University in Poznań.

In total, four molecular markers were amplified: one mitochondrial gene, i.e., COI—the cytochrome oxidase subunit I; three nuclear markers, i.e., 18S rRNA—the small ribosome subunit and 28S rRNA—the large ribosome subunit as well as ITS-2—the internal transcribed spacer-2. The polymerase chain reaction (PCR) amplification was performed according to Kaczmarek et al. [44]. The sequences of primers applied to amplify molecular markers are listed in Table 1. All PCR reactions were conducted in a Biometra TProfessional thermocycler. Prior to the sequencing, the PCR products were treated with the FastAP Alkaline Phosphatase and thermosensitive Exonuclease I (Fermentas, Thermo Scientific, Waltham, MA, USA) according to the manufacturer’s guidelines. Sanger DNA sequencing in both directions was performed by Macrogen (Amsterdam, The Netherlands).

**Table 1.** Primers used for PCR amplification of four DNA molecular markers of *Bryodelphax mareki* sp. nov., *Macrobiotus birendrai* sp. nov. and *Mesobiotus skorackii*.

DNA Fragment	Primer Name/Direction	Primer Sequence (5'-3')	Source
COI	LCO1490/forward HCO2198/reverse	GGTCAACAAATCATAAAGATATTGG TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. [45]
18S rRNA	SSU01_F/forward SSU82_R/reverse	AACCTGGTTGATCCTGCCAGT TGATCCTTCTGCAGGTTACCTAC	Sands et al. [46]
28S rRNA	28SF0001/forward 28SR0990/reverse	ACCCvCynAATTTAAGCATAT CCTTGGTCCGTGTTTCAAGAC	Mironov et al. [47]
ITS-2	ITS3/forward ITS4/reverse	GCATCGATGAAGAACGCAGC TCCTCCGCTTATTGATATGC	White et al. [48]

### 2.6. Comparative Genetic Analysis

Obtained mtDNA and nrDNA sequences were quality checked and consensus sequences were created for individual tardigrades in BioEdit v. 7.2.5 [49]. All COI sequences were translated into amino acid sequences to check against pseudogenes using the EMBOSS-TRANSEQ application [50,51]. The translation was performed with the invertebrate mitochondrial codon table. To verify the homology of the amplified DNA region, Basic Local Alignment Search Tool [52] searches at the National Centre for Biotechnology Information NCBI were applied.

All obtained sequences were deposited in GenBank and the accession numbers are listed in Table 2.

**Table 2.** The GenBank accession numbers of obtained molecular markers of three tardigrade species.

Species	GenBank Accession Numbers (Voucher Numbers of Specimens)			
	COI	18S rRNA	28S rRNA	ITS-2
<i>Bryodelphax mareki</i> sp. nov.	MW655785-87 (CN8.17/S, CN8.25/S, CN8.28/S)	MW680639-40 (CN8.21/S, CN8.25/S)	MW680637-38 (CN8.21/S, CN8.22/S)	NA
<i>Macrobiotus birendrai</i> sp. nov.	MW656266 (CN8.101/S)	MW680641 (CN8.101/S)	MW680644 (CN8.101/S)	MW680418 (CN8.101/S)
<i>Mesobiotus skorackii</i>	MW656257 (CN8.115/S)	MW680642-43 (CN8.22/S, CN8.115/S)	MW680636 (CN8.115/S)	NA

For molecular comparisons, all sequences of the mtDNA and nrDNA fragments of the genera *Bryodelphax*, *Macrobiotus* and *Mesobiotus* were downloaded from the GenBank and trimmed to the same length in BioEdit v. 7.2.5. The COI sequences could be unambiguously aligned without inserting gaps. In turn, the sequences of nrDNA were aligned using ClustalW Multiple Alignment tool [53] implemented in BioEdit v. 7.2.5. with default settings. Uncorrected pairwise distances were calculated using MEGA X [54].

## 3. Results

### Taxonomic Account

Phylum: Tardigrada Doyère, 1840 [55]

Class: Heterotardigrada Marcus, 1927 [56]

Order: Echiniscoidea Richters, 1926 [57]

Family: Echiniscidae Thulin, 1928 [8]

Genus: *Bryodelphax* Thulin, 1928 [8]

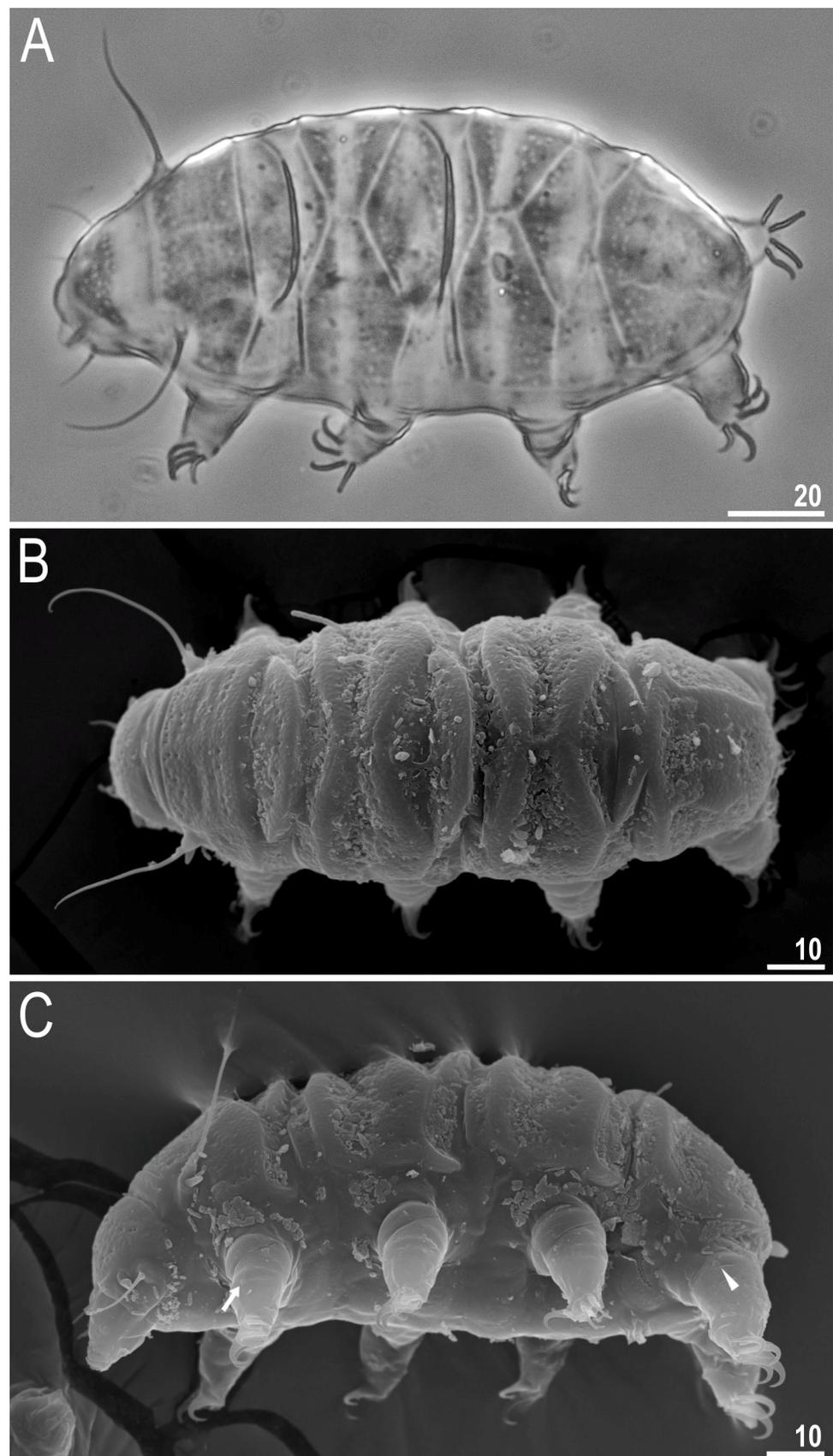
*Bryodelphax mareki* sp. nov.

(Table 3, Figures 1–4)

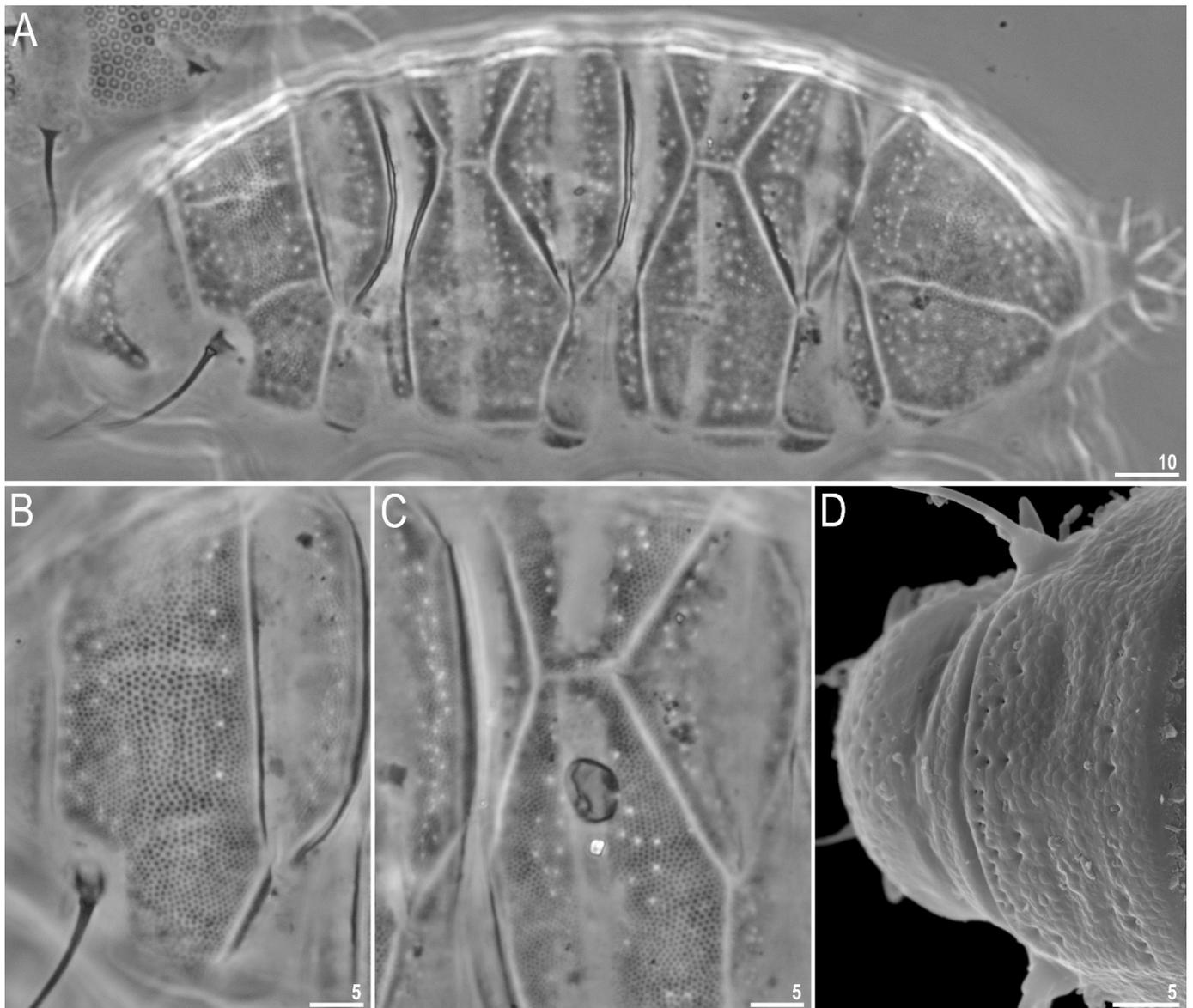
LSID <http://zoobank.org/urn:lsid:zoobank.org:act:F4ACE4EB-4872-43C8-8997-BD40CCBE17EE>

**Table 3.** Measurements (in  $\mu\text{m}$ ) and *sc* values of selected morphological structures of adult females of *Bryodelphax mareki* sp. nov. mounted in Hoyer's medium (N—number of specimens/structures measured; RANGE refers to the smallest and the largest structure among all measured specimens; SD—standard deviation).

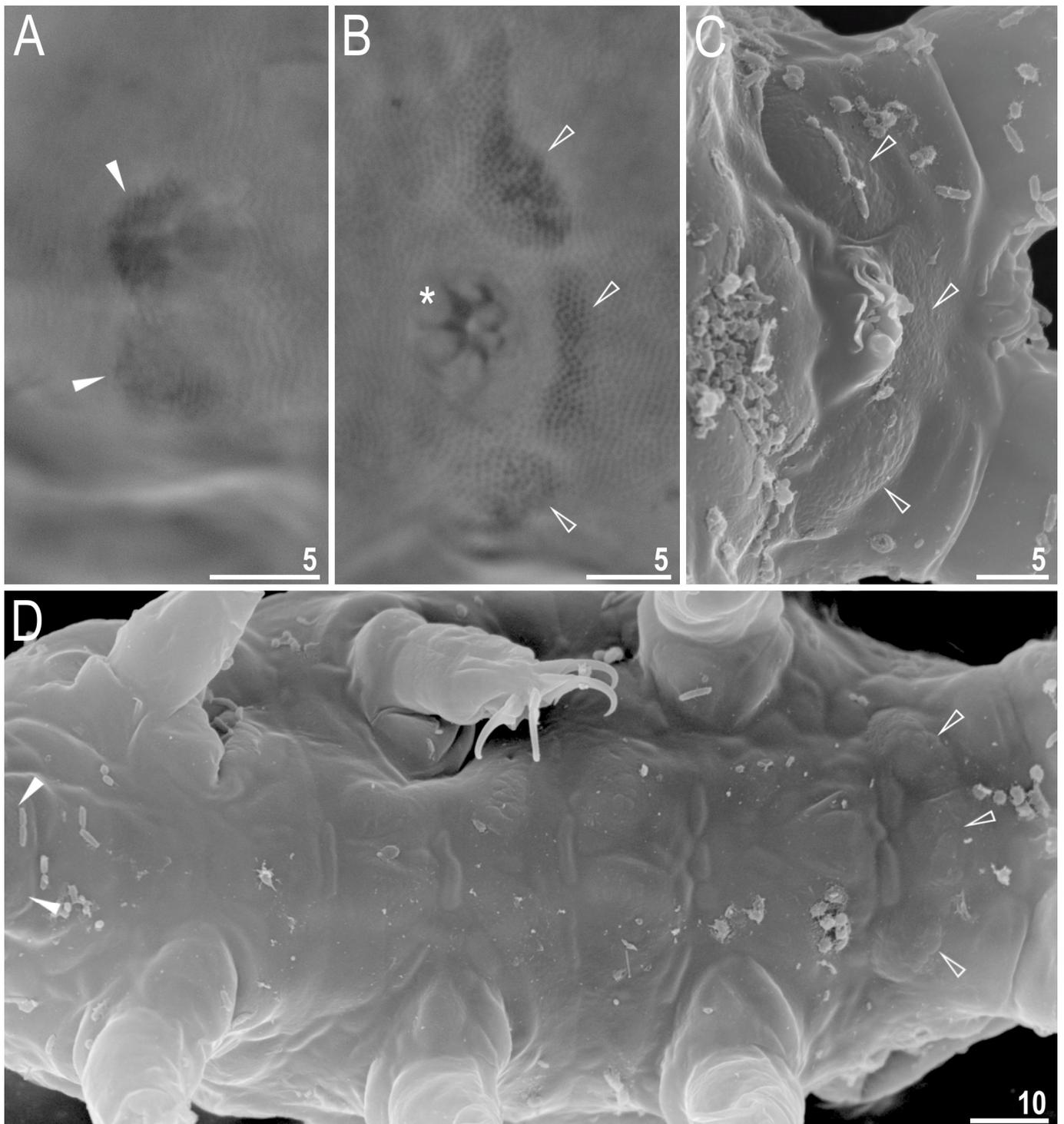
Character	N	Range						Mean		SD		Holotype	
		$\mu\text{m}$		<i>sc</i>		$\mu\text{m}$	<i>sc</i>	$\mu\text{m}$	<i>sc</i>	$\mu\text{m}$	<i>sc</i>		
Body length	16	122	–	181	577	–	780	141	689	15	60	146	749
Scapular plate length	16	18.4	–	24.0		–		20.4	–	1.7	–	19.5	–
Head appendages lengths													
Cirrus <i>internus</i>	16	5.3	–	7.3	26.8	–	34.6	6.2	30.6	0.5	2.7	5.3	27.0
Cephalic papilla	15	4.1	–	5.9	21.7	–	29.1	4.9	24.1	0.5	2.3	4.3	22.0
Cirrus <i>externus</i>	16	11.1	–	13.1	53.4	–	63.0	11.9	58.7	0.7	3.2	11.1	57.0
Clava	12	4.3	–	6.2	20.4	–	33.0	5.2	26.1	0.5	3.7	4.8	24.6
Cirrus <i>A</i>	15	35.2	–	46.7	174.1	–	212.9	39.6	193.6	3.2	10.7	36.5	186.9
Cirrus <i>A</i> /body length ratio	15	25%	–	32%		–		28%	–	2%	–	25%	–
Cirrus <i>int/ext</i> length ratio	16	47%	–	56%		–		52%	–	3%	–	47%	–
Body appendages lengths													
Papilla on leg IV length	14	1.6	–	3.3	8.1	–	14.3	2.3	10.9	0.5	1.9	2.2	11.3
Claw 1 heights													
Branch	16	5.8	–	8.4	30.3	–	42.0	7.6	37.5	0.7	2.8	7.0	35.9
Spur	13	1.3	–	1.7	5.4	–	8.2	1.5	7.2	0.1	0.8	1.4	7.2
Spur/branch length ratio	13	16%	–	21%		–		18%	–	2%	–	0	–
Claw 2 heights													
Branch	16	6.7	–	8.6	30.8	–	42.6	7.6	37.7	0.5	3.6	7.5	38.3
Spur	14	1.0	–	1.5	5.2	–	7.2	1.3	6.6	0.1	0.7	1.4	7.2
Spur/branch length ratio	14	15%	–	20%		–		18%	–	1%	–	0	–
Claw 3 heights													
Branch	16	5.8	–	8.6	30.6	–	45.1	7.5	37.3	0.8	4.0	7.0	35.6
Spur	12	1.2	–	1.5	5.4	–	7.5	1.4	6.6	0.1	0.8	1.3	6.7
Spur/branch length ratio	12	16%	–	20%		–		18%	–	1%	–	0	–
Claw 4 lengths													
Branch	16	7.3	–	9.9	38.3	–	48.2	8.9	43.8	0.6	3.7	8.8	45.2
Spur	13	1.4	–	1.9	6.3	–	8.9	1.6	7.8	0.1	0.7	1.6	8.0
Spur/branch height ratio	13	15%	–	21%		–		18%	–	2%	–	0	–



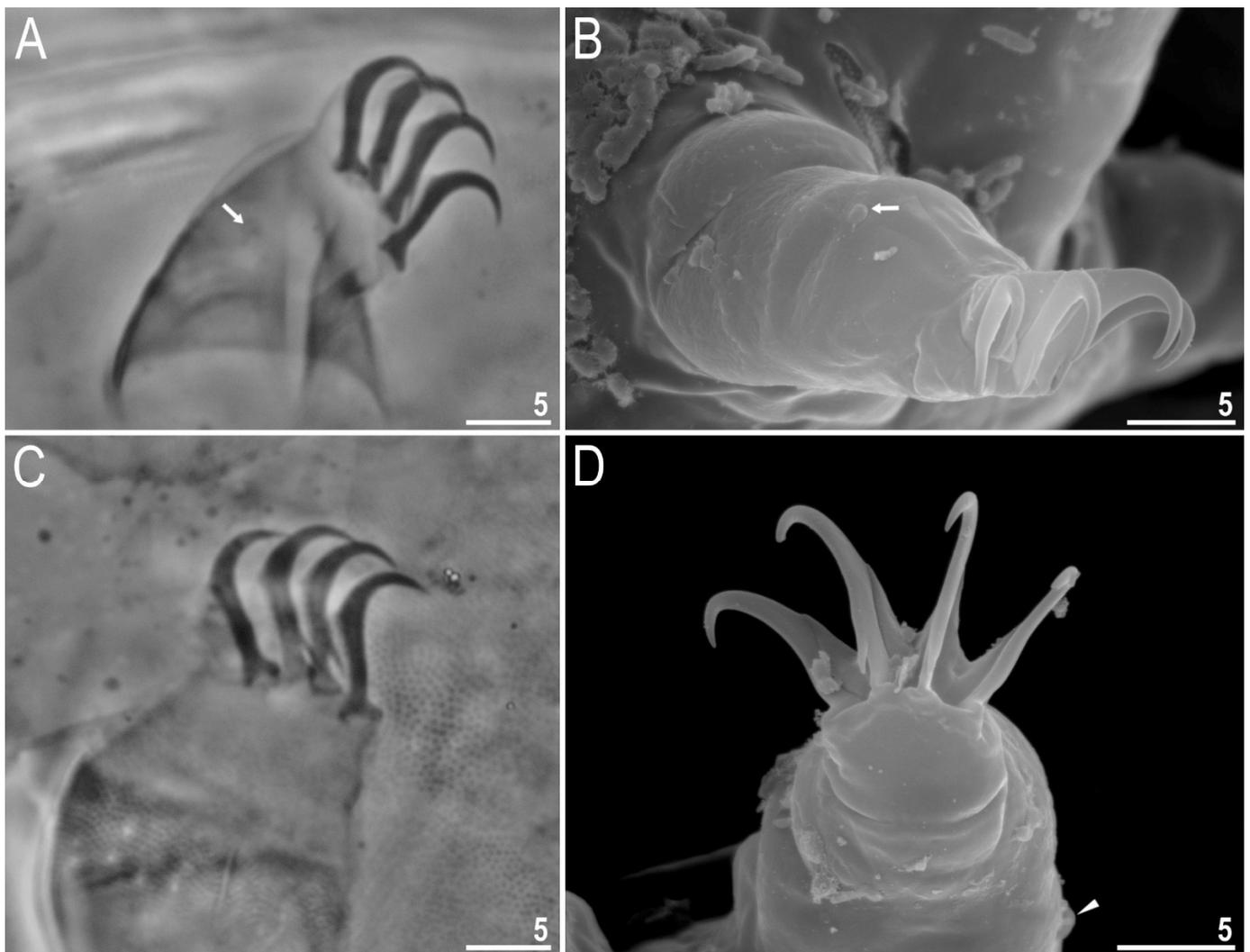
**Figure 1.** *Bryodelphax mareki* sp. nov.: habitus: (A) dorsal projection (holotype, PCM); (B) dorsal projection (SEM); (C) lateral projection; arrow indicates papilla-like structure on leg I, arrowhead indicates papilla on leg IV (SEM). Scale bars in  $\mu\text{m}$ .



**Figure 2.** *Bryodelphax mareki* sp. nov.: (A) dorso-lateral view of the details of dorsal plates (paratype, PCM); (B) close up of the head plate (holotype, PCM); (C) close up of the pair plates II (holotype, PCM); (D) close up of the head and scapular plates (SEM). Scale bars in  $\mu\text{m}$ .



**Figure 3.** *Bryodelphax mareki* sp. nov.: ventral plates: (A) two ventral plates under head (filled arrowheads) (paratype, PCM); (B,C) three ventral plates around gonophore (empty arrowheads); asterisk indicates gonophore (paratypes, PCM and SEM, respectively); (D) ventral projection visible in SEM; filled arrowheads indicate two ventral plates under head, empty arrowheads indicate ventral plates around gonophore. Scale bars in  $\mu\text{m}$ .



**Figure 4.** *Bryodelphax mareki* sp. nov.: (A,B) claws of leg I with visible papilla-like structure (arrow) (paratypes, PCM and SEM, respectively); (C,D) claws of leg IV; arrowhead indicates papilla on leg IV (paratypes, PCM and SEM, respectively). Scale bars in  $\mu\text{m}$ .

*Type Locality:* 51°24'21" N, 116°14'27" W, 1900 m asl, Canada, Alberta, Banff National Park, near east end of the Louise Lake, moss on stone, May 2019, leg. Milena Roszkowska and Łukasz Kaczmarek.

*Material examined:* The 74 animals, i.e., holotype + 73 paratypes (females: 37; undefined sex: 34 and 2 exuviae) mounted on microscope slides in Hoyer's medium, 40 animals prepared for SEM and 20 animals prepared for molecular analyses (not included in the type series). However, DNA sequences were obtained from only five specimens (exoskeletons) which was later mounted on microscope slide in Hoyer's medium and included into type series.

*Type depositories:* Holotype (CN8.62) and 76 paratypes (slides: CN8.\*, where the asterisk can be substituted by any of the following numbers: 46–55, 65–66, 17/S, 21/S, 22/S, 25/S, 28/S) are deposited at the Department of Animal Taxonomy and Ecology, Institute of Environmental Biology, Adam Mickiewicz University in Poznań, Uniwersytetu Poznańskiego 6, 61-614 Poznań, Poland. Two paratypes (two females; slides CN8.63 and CN8.64) are deposited in the Natural History Museum of Denmark, University of Copenhagen.

*Etymology:* The authors would like to dedicate this species to famous biochemist and last author's friend—Professor Marek Michalak, Faculty of Medicine and Dentistry, Biochemistry Department, University of Alberta, Edmonton, AB, Canada.

#### Description of the new species

Adult females (measurements and statistics in Table 3). Body light yellow in live specimens (transparent after mounting in Hoyer's medium) (Figure 1A–C), eyes absent or not visible after mounting on microscope slides. Small and conical primary and secondary clavae present. Cirri *internus* and *externus* with poorly developed cirrophores. Cirri *internus* always shorter than cirri *externus*. Cirri A of a typical length for *Bryodelphax*, i.e., up to 25% of the total body length. Only lateral appendages cirri A present apart from head appendages.

Dorsal sculpture, visible in PCM, composed of intra-cuticular pillars (visible as dark dots/granules) and pores (visible as white dots) (Figure 2A–C). The cuticular pillars (granules) on scapular plate 0.6–1.8 µm in diameter, on caudal plate 0.6–1.6 µm in diameter and on other plates 0.6–1.4 µm in diameter. Pores large and easily detectable (Figure 2A–D), distributed unevenly on scapular plate (0.3–0.6 µm in diameter; 0–14 pores/100 µm<sup>2</sup>,  $\bar{x}$  = 7.5,  $N$  = 10); on caudal plate (0.6–1.0 µm in diameter; 0–19 pores/100 µm<sup>2</sup>,  $\bar{x}$  = 4.44,  $N$  = 10) and on other plates (0.3–0.8 µm in diameter; 1–16 pores/100 µm<sup>2</sup>,  $\bar{x}$  = 6.3,  $N$  = 10). Median plates 1 and 2 divided by smooth transverse stripe, median plate 3 undivided. Median plate 2 largest among all median plates. Paired plates 1 and 2 also divided transversely into two parts by smooth stripes.

Ventral side with three rows of greyish plates (formula: III:2-2-1). First row with two plates just below the head (Figure 3A,D, filled arrowheads). Three genital plates surrounding the gonopore (two lateral, in line with the gonopore) and the third one situated posteriorly to the gonopore (Figure 3B–D, empty arrowheads)).

Papilla-like structure on leg I hardly visible under PCM but visible in SEM (Figures 1C and 4A,B, arrow). Papillae on leg IV present (Figures 1C and 4D, arrowhead). Dentate collar absent on leg IV (Figure 1A–C). All claws slender, claws IV always slightly longer than claws I–III. External claws smooth, internal ones with a small spur pointing downward and placed very close to the claw bases (Figure 4A–D). The female gonopore with the typical six-petal rosette.

**Males and Juveniles.** Not found.

#### DNA sequences

**COI:** three sequences; 584–672 bp;

**18S rRNA:** two sequences; 528 bp long;

**28S rRNA:** two sequences; 671 bp long.

**Differential diagnosis.** Presence of ventral plates attributes *Bryodelphax mareki* sp. nov. to the *weglarskae* group. Within this group, only *Bry. amphoterus* (Durante Pasa and Maucci [58]), *Bry. maculatus* Gąsiorek, Stec, Morek, Marnissi and Michalczyk [58] and *Bry. nigripunctatus* Degma, Gąsiorek, Vončina and Michalczyk [30] have a reduced number of ventral plate rows to two or three, as in the new species [30]. Adult females of *Bry. mareki* sp. nov. differ from:

*Bry. amphoterus*, known only from Croatia and Greece (McInnes [59]), by different formula of ventral plates (III:2-2-1 in the new species vs. II:2-2 in *Bry. amphoterus*), presence of papilla-like structure on leg I and papillae on leg IV and absence of dentate collar on leg IV.

*Bry. maculatus*, known only from Tunisia and Greece (Gąsiorek et al. [60]), by higher *sc* of clava (20.4–33.0 in the new species vs. 11.5–19.1 in *Bry. maculatus*), longer cirrus A (35.2–46.7 µm in the new species vs. 27.3–34.9 µm in *Bry. maculatus*), higher *sc* of cirrus A (174.1–212.9 in the new species vs. 114.8–152.5 in *Bry. maculatus*) and absence of dentate collar on leg IV.

*Bry. nigripunctatus* known only from Spain (Gąsiorek et al. [30]), by absence of epicuticular granules, longer clava (4.3–6.2 µm in the new species vs. 2.7–3.1 µm in *Bry. nigripunctatus*), higher *sc* of clava (20.4–33.0 in the new species vs. 12.3–16.9 in *Bry. nigripunctatus*) and presence of papilla-like structure on leg I.

### Genetic variability

Aligned sequences (obtained in present study and downloaded from GenBank) were trimmed to 591, 498 and 700 bp for COI (four sequences; two species), 18S rRNA (eight sequences; four species and two sequences of *Bryodelphax* sp.) and 28S rRNA (14 sequences; seven species and two sequences of *Bryodelphax* sp.) molecular markers, respectively. Only sequences of *Bryodelphax* downloaded from GenBank that coincided with our four aforementioned molecular markers were selected.

In the case of the COI molecular marker, only three sequences of *Bry. parvulus* [8] were available in GenBank. Analysis of the p-distances between our sequences (GenBank accession numbers: MW655785-87) and three sequences of *Bry. parvulus* was from 16% (GenBank accession numbers: JX683827, JX683826, unpublished) to 18% (GenBank accession number: HM193405 [61]). In the case of the 18S rRNA molecular marker (GenBank accession numbers of our sequences: MW680639-40), no genetic differences were observed when compared with *Bry. parvulus* (GenBank accession numbers: HM193371 [61]; JX676189, [62]) and p-distance between other sequences, i.e., *Bryodelphax* sp. (GenBank accession numbers: EU266963 and EF632433 [63]) and *Bry. tatrensis* [64] (GenBank accession numbers: JX676188 and JX676190 [62]) was 0.01%. The ranges of uncorrected genetic p-distances between our 28S rRNA sequences (GenBank accession numbers: MW680637-38) and the most similar *Bry. cf. parvulus* (GenBank accession number: MT333466 [30]) was 0.01% and the least similar *Bryodelphax* sp. (GenBank accession numbers: MH414964 [65]) was 0.16% (see Supplementary Materials—SM1).

**Class:** Eutardigrada Richters, 1926 [57]

**Order:** Parachela Schuster, Nelson, Grigarick and Christenberry, 1980 [66]

**Superfamily:** Macrobiotioidea Thulin, 1928 [8]

**Family:** Macrobiotidae Thulin, 1928 [8]

**Genus:** *Macrobiotus* C.A.S. Schultze, 1834 [11]

***Macrobiotus birendrai* sp. nov.**

(Tables 4 and 5, Figures 5–8)

LSID <http://zoobank.org/urn:lsid:zoobank.org:act:5617E45B-865A-42DA-A70A-DB6FE1A52163>

**Table 4.** Measurements (in  $\mu\text{m}$ ) and *pt* values of selected morphological structures of individuals of *Macrobiotus birendrai* sp. nov. mounted in Hoyer's medium (N—number of specimens/structures measured; RANGE refers to the smallest and the largest structure among all measured specimens; SD—standard deviation, *pt*—ratio of the length of a given structure to the length of the buccal tube expressed as a percentage).

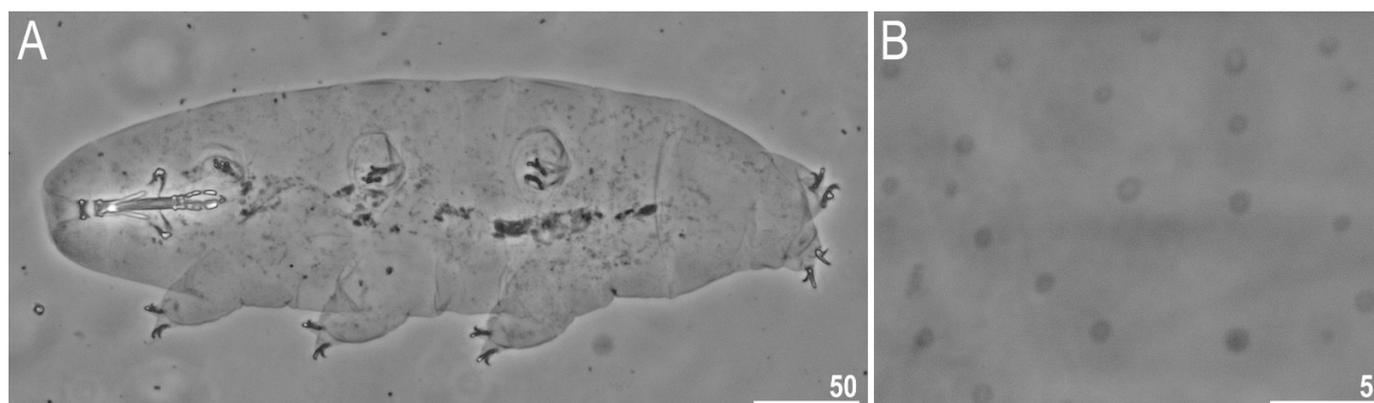
Character	N	Range				Mean		SD		Holotype			
		$\mu\text{m}$	$\mu\text{m}$	$\mu\text{m}$	$\mu\text{m}$	$\mu\text{m}$	$pt$	$\mu\text{m}$	$pt$	$\mu\text{m}$	$pt$		
Body length	15	271	–	589	815	–	1291	449	1055	97	133	368	978
<b>Buccopharyngeal tube</b>													
Buccal tube length	16	33.2	–	52.6	–	–	–	42.7	–	5.9	–	37.7	–
Stylet support insertion point	16	26.2	–	43.0	78.0	–	82.8	34.3	80.2	5.2	1.6	29.6	78.7
Buccal tube external width	16	5.2	–	8.4	13.5	–	17.0	6.6	15.3	1.0	1.1	5.5	14.7
Buccal tube internal width	16	3.4	–	5.6	8.7	–	11.9	4.4	10.2	0.8	1.0	3.8	10.0
Ventral lamina length	13	19.9	–	32.9	56.0	–	66.2	25.7	60.9	4.2	3.2	22.9	60.8
<b>Placoid lengths</b>													
Macroplacoid 1	16	8.2	–	16.0	23.0	–	31.6	11.3	26.2	2.2	2.6	8.7	23.0
Macroplacoid 2	16	6.0	–	11.2	17.2	–	22.0	8.1	18.9	1.6	1.5	6.5	16.3
Microplacoid	16	2.4	–	4.9	6.5	–	9.6	3.5	8.0	0.8	0.9	2.8	7.4
Macroplacoid row	16	16.3	–	30.2	49.1	–	57.9	22.6	52.6	4.4	3.4	18.8	49.9
Placoid row	16	19.2	–	35.2	57.9	–	67.9	26.9	62.7	4.9	3.4	23.2	61.5
<b>Claw 1 heights</b>													
External primary branch	16	8.5	–	13.2	20.6	–	29.8	10.8	25.3	1.6	2.3	10.0	26.5
External secondary branch	16	6.1	–	10.3	14.5	–	22.1	8.1	19.0	1.4	2.2	7.8	20.7
Internal primary branch	16	8.2	–	12.7	20.3	–	27.7	10.3	24.2	1.5	1.8	9.3	24.8
Internal secondary branch	16	6.2	–	10.7	16.7	–	24.3	8.4	19.6	1.4	2.3	7.8	20.8

Table 4. Cont.

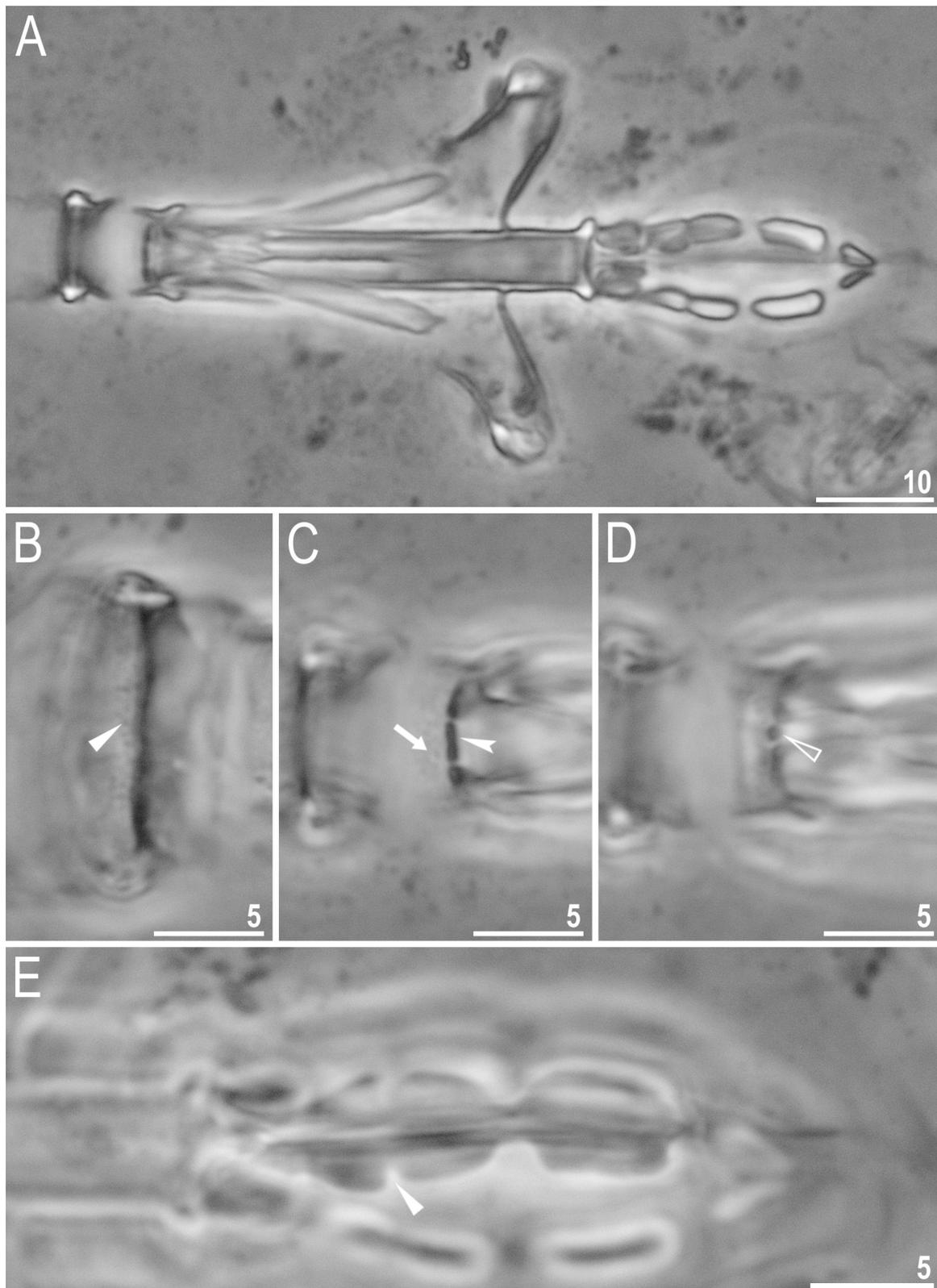
Character	N	Range				Mean		SD		Holotype			
		$\mu\text{m}$		$\mu\text{m}$									
<b>Claw 2 heights</b>													
External primary branch	16	8.1	–	14.0	23.8	–	29.3	11.5	26.9	1.7	1.6	10.3	27.2
External secondary branch	16	7.0	–	11.7	17.5	–	23.9	9.0	21.1	1.5	1.9	7.5	19.8
Internal primary branch	16	8.6	–	13.6	23.0	–	30.7	11.1	26.0	1.6	2.1	11.1	29.5
Internal secondary branch	16	6.8	–	11.0	16.5	–	23.6	8.5	20.0	1.3	2.2	8.8	23.3
<b>Claw 3 heights</b>													
External primary branch	16	8.7	–	14.8	24.9	–	31.7	11.7	27.4	1.7	2.1	10.4	27.5
External secondary branch	16	7.1	–	11.7	17.5	–	25.2	9.2	21.5	1.5	1.7	7.8	20.8
Internal primary branch	16	8.2	–	13.5	22.7	–	30.3	10.9	25.5	1.7	1.9	9.7	25.8
Internal secondary branch	16	6.5	–	11.1	18.0	–	23.2	8.8	20.6	1.5	1.7	8.3	21.9
<b>Claw 4 lengths</b>													
Anterior primary branch	16	9.5	–	15.8	23.9	–	34.9	12.5	29.2	1.9	2.9	11.7	30.9
Anterior secondary branch	16	6.7	–	13.0	18.3	–	28.7	9.5	22.2	1.7	2.7	8.9	23.5
Posterior primary branch	16	8.4	–	14.2	23.7	–	32.2	12.1	28.3	1.5	2.4	11.6	30.8
Posterior secondary branch	16	6.5	–	12.0	18.3	–	24.3	9.2	21.5	1.7	1.9	8.3	22.1

**Table 5.** Measurements (in  $\mu\text{m}$ ) of selected morphological structures of eggs of *Macrobiotus birendrai* sp. nov. mounted in Hoyer's medium (N—number of specimens/structures measured; RANGE refers to the smallest and the largest structure among all measured eggs; SD—standard deviation).

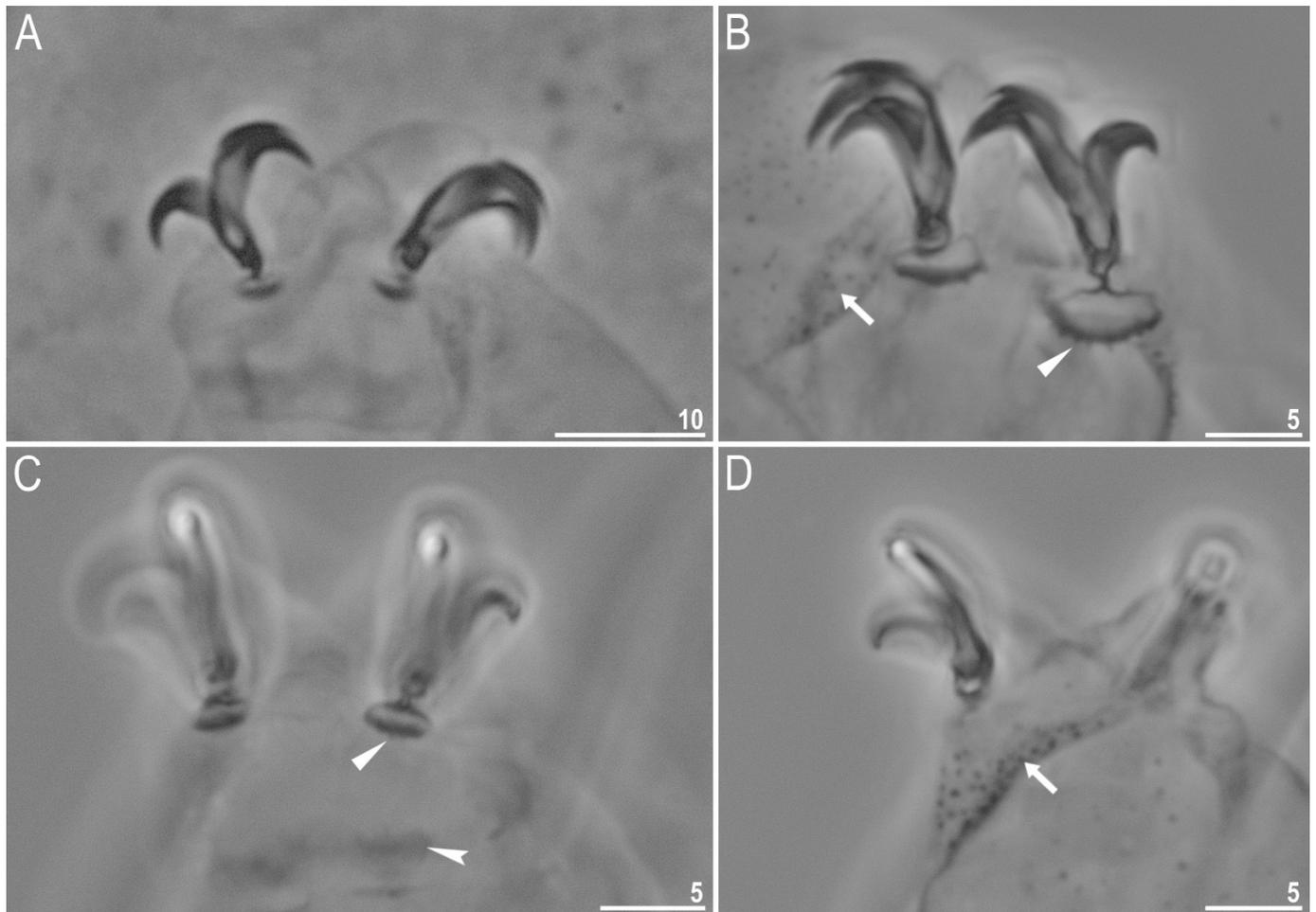
Character	N	Range	Mean	SD
Egg bare diameter	5	74.9–86.9	87.2	7.6
Egg full diameter	5	87.8–103.9	102.4	9.3
Process height	33	5.9–9.4	7.9	1.1
Process base width	33	5.5–9.0	6.6	0.8
Process base/height ratio	33	65–100%	84%	10%
Terminal disc width	33	3.2–6.1	4.1	0.7
Inter-process distance	33	1.0–3.3	2.1	0.7
Number of processes on the egg circumference	5	22–26	23.6	2.0



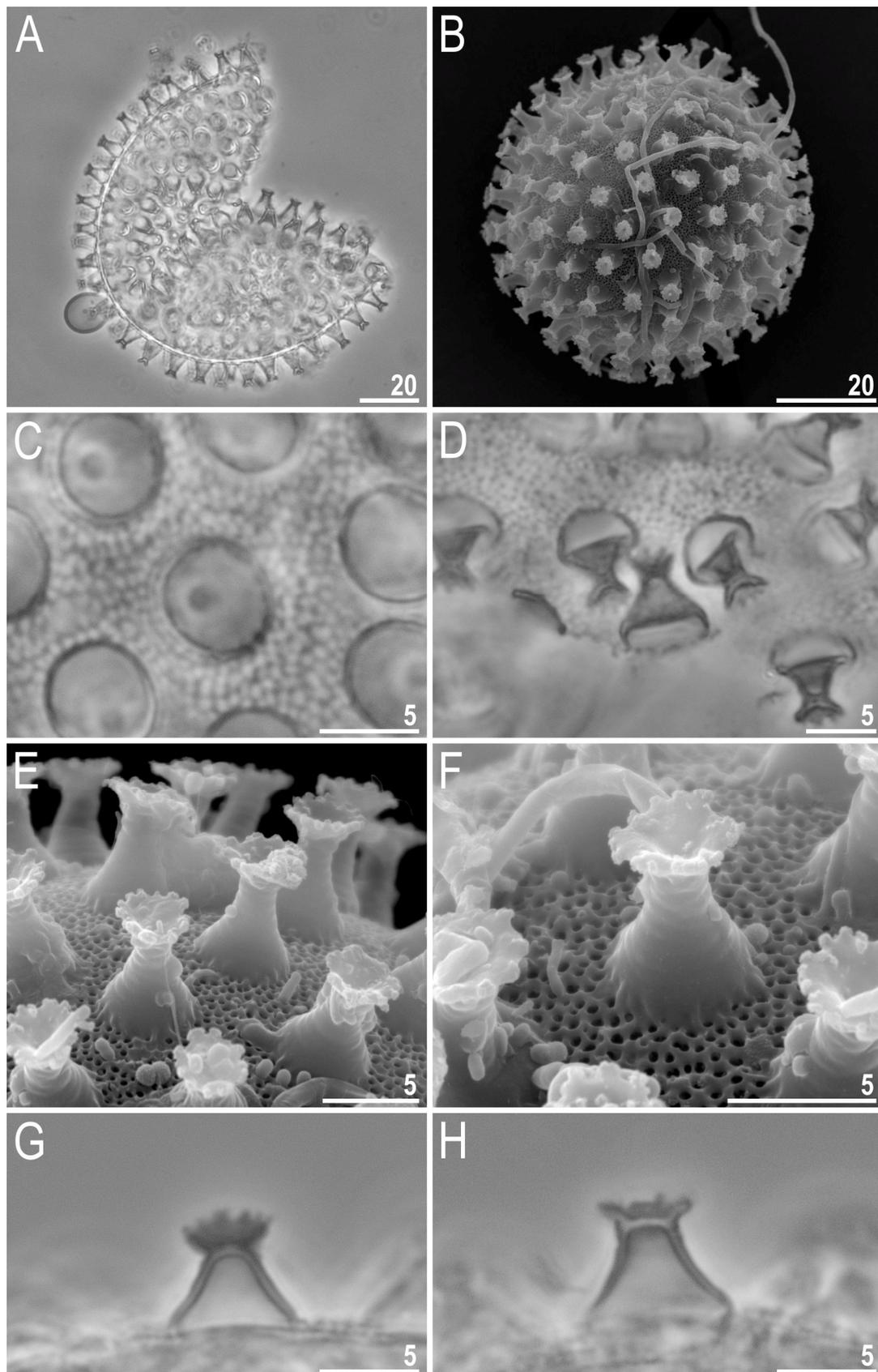
**Figure 5.** *Macrobiotus birendrai* sp. nov.: (A) dorso-ventral projection (holotype); (B) cuticular pores on dorsal side of the body (paratype). All PCM. Scale bars in  $\mu\text{m}$ .



**Figure 6.** *Macrobiotus birendrai* sp. nov.: bucco-pharyngeal apparatus (dorso-ventral projection): (A) general view (paratype); (B) oral cavity armature with filled arrowhead indicating teeth of the first band (paratype); (C) oral cavity armature with arrow indicating teeth of the second band and indented filled arrowhead indicating dorsal teeth of the third band (paratype); (D) oral cavity armature with empty arrowhead indicating ventral teeth of the third band (paratype); (E) ventral placoids; the filled arrowhead indicates a first macroplacoid with central constriction (holotype). All PCM. Scale bars in  $\mu\text{m}$ .



**Figure 7.** *Macrobiotus birendrai* sp. nov.: (A) claws III (paratype); (B) claws IV with dentate lunulas (arrowhead); arrow indicates granulation on legs IV (paratype); (C) lunulas under claws III with small teeth; indented arrowhead indicates cuticular bar under claws (paratype); (D) granulation on leg III (arrow) (holotype). All PCM. Scale bars in µm.



**Figure 8.** *Macrobiotus birendrai* sp. nov.: eggs: (A,B) egg chorion (PCM and SEM, respectively); (C) the surface between egg processes visible in PCM; (D) egg processes visible in PCM; (E,F) egg surface and processes visible in SEM; (G,H) egg processes visible in PCM. Scale bars in  $\mu\text{m}$ .

*Type Locality:* 51°24'21" N, 116°14'27" W, 1900 m asl, Canada, Alberta, Banff National Park, near east end of the Louise Lake, moss on stone, May 2019, leg. Milena Roszkowska and Łukasz Kaczmarek.

*Material examined:* The 57 specimens, i.e., holotype (slide: CN8.43) + 56 paratypes (adults: 44 and eggs: 12) were mounted on microscope slides in Hoyer's medium, four eggs prepared for SEM and five animals prepared for molecular analyses (not included in type series). However, DNA sequences were obtained from one female specimen (exoskeleton) which was later mounted on microscope slide in Hoyer's medium and included into type series.

*Type depositories:* Holotype (CN8.43) and 57 paratypes (slides: CN8.\*, where the asterisk can be substituted by any of the following numbers: 3, 5–7, 15–17, 21, 29–33, 39, 40, 42–45, 101/S) are deposited at the Department of Animal Taxonomy and Ecology, Institute of Environmental Biology, Adam Mickiewicz University in Poznań, Uniwersytetu Poznańskiego 6, 61-614 Poznań, Poland. Six paratypes (five adults and one egg; slides CN8.41 and CN8.28) are deposited in the Natural History Museum of Denmark, University of Copenhagen.

*Etymology:* The first author would like to dedicate this species to her father—Birendra Prasad Lal Karna.

#### *Description of the new species.*

Adults (measurements and statistics in Table 4). Body transparent after fixation in Hoyer's medium, eyes present in all fixed specimens (Figure 5A). Entire cuticle covered with conspicuous round and lenticular pores (0.6–1.8 µm in diameter) distributed randomly (Figure 5B). However, larger pores present, on dorsal side, at the anterior and posterior part of the body. Bucco-pharyngeal apparatus of the *Macrobotus* type, with ventral lamina and 10 peribuccal lamellae (Figure 6A). Mouth antero-ventral. Oral cavity armature of the *hufelandi* type, with first and the second band composed of numerous minute teeth (visible as granules in PCM) and third composed of three dorsal and three ventral transverse ridges (Figure 6B–D). Pharyngeal bulb spherical with triangular apophyses, two rod-shaped macroplacoids and a triangular microplacoid. Macroplacoid length sequence  $2 < 1$  (Figure 6A,E). The first macroplacoid with central constriction (Figure 6E, arrowhead), second with sub-terminal constriction. Claws of the *hufelandi* type (Figure 7A,B). Primary branches with distinct accessory points. Lunules under claws I–III with hardly visible teeth (visible only in bigger specimens) (Figure 7C, arrowhead) and dentate under claws IV (Figure 7B, arrowhead). Thin single continuous cuticular bars under claws I–III present (Figure 7C, indented arrowhead). Easily visible granulation present on legs I–IV (Figure 7B,D, arrows).

Eggs (measurements and statistics in Table 5). Eggs spherical, ornamented and laid freely with egg chorion of the *hufelandi* type (Figure 8A,B). Pores of egg surface mesh circular, similar in size and rather small, i.e., 0.2–0.8 µm in diameter (Figure 8C,E,F). Processes in the shape of inverted concave cups with terminal discs (Figure 8D–H). Terminal discs concave with serrated margins or with small irregular teeth (Figure 8D–H).

#### **DNA sequences**

We obtained good quality sequences for the applied molecular markers:

**COI:** single sequence; 609 bp long;

**18S rRNA:** single sequence; 553 bp long;

**28S rRNA:** single sequence; 721 bp long;

**ITS-2:** single sequence; 350 bp long.

**Differential diagnosis.** Based on egg processes morphology, the new species is most similar to *Mac. canaricus* Stec et al. [67], *Mac. hanna*e Nowak and Stec [68], *Mac. crustulus* Stec et al. [12], *Mac. joannae* Pilato and Binda [69], *Mac. kamilae* Coughlan and Stec [70], *Mac. madegassus* Maucci [71], *Mac. noemiae* Roszkowska and Kaczmarek [72], *Mac. noongaris* Coughlan and Stec [70], *Mac. papei* Stec et al. [73], *Mac. paulinae* Stec et al. [16], *Mac. polypiformis* Roszkowska et al. [38] and *Mac. porifini* Kuzdrowska et al. [39], but differs specifically from:

**Mac. canaricus** Stec, Krzywański and Michalczyk, 2018, known only from the type locality on Canary Islands [67], by a different oral cavity armature (*hufelandi* type in the new species vs. *maculatus* type in *Mac. canaricus*), lunules I-III with hardly visible teeth, larger buccal tube internal width (3.4–5.6  $\mu\text{m}$  in the new species vs. 2.1–3.3  $\mu\text{m}$  in *Mac. canaricus*), higher *pt* of placoid row (57.9–67.9 in the new species vs. 42.6–55.3 in *Mac. canaricus*), larger cuticular pores (0.7–1.8  $\mu\text{m}$  in the new species vs. 0.4–0.7  $\mu\text{m}$  in *Mac. canaricus*), different egg shell surface (porous shell in the new species vs. mesh shell in *Mac. canaricus*) and absence of granulation on terminal discs of the egg process.

**Mac. crustulus** Stec, Dudziak and Michalczyk, 2020, known only from the type locality in French Guiana [12], by a different oral cavity armature (*hufelandi* type in the new species vs. *lissostomus* type in *Mac. crustulus*), lunules I-III with hardly visible teeth, higher *pt* of stylet support insertion points (78.0–82.8 in the new species vs. 69.3–72.7 in *Mac. crustulus*), larger buccal tube internal width (3.4–5.6  $\mu\text{m}$  in the new species vs. 1.6–2.7  $\mu\text{m}$  in *Mac. crustulus*), higher *pt* of buccal tube external and internal width (13.5–17.0 and 8.7–11.9, respectively, in the new species vs. 9.3–12.2 and 5.1–6.5, respectively, in *Mac. crustulus*), higher *pt* of ventral lamina (56.0–66.2 in the new species vs. 46.9–55.8 in *Mac. crustulus*), different egg shell surface (porous shell in the new species vs. mesh shell in *Mac. crustulus*), absence of granulation on convex central area of terminal discs and lower number of processes on the egg circumference (22–26 in the new species vs. 26–34 in *Mac. crustulus*).

**Mac. hanna**e Nowak and Stec, 2018, known only from the type locality in Poland [68], by lunules I-III with hardly visible teeth, larger cuticular pores (0.6–1.8  $\mu\text{m}$  in the new species vs. up to 0.55  $\mu\text{m}$  in *Mac. hanna*e), smaller distance between egg processes (1.0–3.3  $\mu\text{m}$  in the new species vs. 4.0–8.1  $\mu\text{m}$  in *Mac. hanna*e), smaller egg bare diameter (74.9–86.9  $\mu\text{m}$  in the new species vs. 88.6–109.2  $\mu\text{m}$  in *Mac. hanna*e) and absence of granules inside pores around egg processes.

**Mac. joannae** Pilato and Binda, 1983, known only from the type locality in Australia (Pilato and Binda [69]; see also comments in Nowak and Stec [68]), by lower average *pt* of stylet support insertion points (80.2 in the new species vs. 81.9 in *Mac. joannae*), smaller size of macroplacoid 1 and microplacoid (8.2–16.0 and 2.4–4.9  $\mu\text{m}$ , respectively, in the new species vs. 19.3 and 6.4  $\mu\text{m}$ , respectively, in *Mac. joannae* in specimen of body length 400  $\mu\text{m}$ ), smaller size of claws I and II external and internal primary branch (I. 8.5–13.2 and 8.2–12.7  $\mu\text{m}$ , respectively; II. 8.1–14.0 and 8.6–13.6  $\mu\text{m}$ , respectively, in the new species vs. I. 25.0 and 23.5  $\mu\text{m}$ , respectively; II. 27.0 and 24.5  $\mu\text{m}$ , respectively in *Mac. joannae* in specimen of body length 400  $\mu\text{m}$ ) and smaller size of claw IV anterior and posterior primary branch (9.5–15.8 and 8.4–14.2  $\mu\text{m}$ , respectively, in the new species vs. 28.0 and 27.0  $\mu\text{m}$ , respectively, in *Mac. joannae* in specimen of body length 400  $\mu\text{m}$ ).

**Mac. kamilae** Coughlan and Stec, 2019, known only from the type locality in India [70], by a different oral cavity armature (*hufelandi* type in the new species vs. *patagonicus* type in *Mac. kamilae*), lunules I-III with hardly visible teeth, higher *pt* of stylet support insertion points (78.0–82.8 in the new species vs. 71.6–75.9 in *Mac. kamilae*), shorter claws (for details see Table 4 in this paper and Table 4 in Coughlan and Stec [65]), absence of body granulation and absence of scattered granules on the terminal discs of egg process.

**Mac. madegassus** Maucci, 1993, known only from the type locality in Madagascar [71], by presence of eyes, different oral cavity armature (*hufelandi* type in the new species vs. *maculatus* type in *Mac. madegassus*), presence of cuticular pores, lunules I-III with hardly visible teeth, longer buccal tube (33.2–52.6  $\mu\text{m}$  in the new species vs. up to 30.0  $\mu\text{m}$  in *Mac. madegassus*), larger buccal tube external width (5.2–8.4  $\mu\text{m}$  in the new species vs. up to 2.1  $\mu\text{m}$  in *Mac. madegassus*), higher *pt* of buccal tube external width (13.5–17.0 in the new species vs. 7.0 in *Mac. madegassus* in specimen of body length 316  $\mu\text{m}$ ), longer macroplacoids (I: 8.2–16.0  $\mu\text{m}$  and II: 6.0–11.2  $\mu\text{m}$  in the new species vs. I: up to 6.4  $\mu\text{m}$  and II: up to 3.6  $\mu\text{m}$  in *Mac. madegassus* in specimen of body length 316  $\mu\text{m}$ ), stylet supports inserted in more caudal position (26.2–43.0  $\mu\text{m}$  in the new species vs. up to 20.4  $\mu\text{m}$  in *Mac. madegassus* in specimen of body length 316  $\mu\text{m}$ ), higher *pt* of stylet support insertion

points (78.0–82.8 in the new species vs. up to 68.0 in *Mac. madegassus* in specimen of body length 316  $\mu\text{m}$ ) and lower number of processes on the egg circumference (20–26 in the new species vs. 30–34 in *Mac. madegassus*).

***Mac. noemiae* Roszkowska and Kaczmarek, 2019**, known only from the type locality in Spain [72], by a different oral cavity armature (*hufelandi* type in the new species vs. *patagonicus* type in *Mac. noemiae*), lunules I–III with hardly visible teeth, higher *pt* of ventral lamina (56.0–66.2 in the new species vs. 48.4–55.7 in *Mac. noemiae*), higher *pt* of macroplacoid row and placoid row (49.1–57.9 and 57.9–67.9, respectively, in the new species vs. 39.2–47.1 and 47.1–57.9, respectively, in *Mac. noemiae*), smaller egg full and bare diameter (87.8–103.9 and 74.9–86.9  $\mu\text{m}$ , respectively, in the new species vs. 118.5–123.5 and 100.6–105.7  $\mu\text{m}$ , respectively, in *Mac. noemiae*), lower number of processes on the egg circumference (20–26 in the new species vs. 30–34 in *Mac. noemiae*), presence of discs on egg processes and absence of filaments on apical part of egg processes.

***Mac. noongaris* Coughlan and Stec, 2019**, known only from the type locality in Australia [70], by a different oral cavity armature (*hufelandi* type in the new species vs. *patagonicus* type in *Mac. noongaris*), lunules I–III with hardly visible teeth, larger maximum size of the cuticular pores (up to 1.8  $\mu\text{m}$  in the new species vs. up to 0.8  $\mu\text{m}$  in *Mac. noongaris*), absence of scattered granulation on the terminal discs of the egg processes, higher mean of egg bare diameter and egg full diameter (82.9 and 96.3  $\mu\text{m}$ , respectively, in the new species vs. 70.7 and 82.1  $\mu\text{m}$ , respectively, in *Mac. noongaris*), larger mean process height, process base width and terminal disc width (7.9, 6.6 and 4.1  $\mu\text{m}$ , respectively, in the new species vs. 6.2, 5.0 and 3.3  $\mu\text{m}$ , respectively, in *Mac. noongaris*), and smaller mean inter processes distance (2.1  $\mu\text{m}$  in the new species vs. 3.4  $\mu\text{m}$  in *Mac. noongaris*).

***Mac. papei* Stec, Kristensen and Michalczyk, 2018** known only from type locality in Tanzania [73], by a different oral cavity armature (*hufelandi* type in the new species vs. *patagonicus* type in *Mac. papei*), lunules I–III with hardly visible teeth, absence of patches of cuticular granulation on the internal surface of legs I–III, higher *pt* of macroplacoid II (17.2–22.0 in the new species vs. 10.3–16.2 in *Mac. papei*), absence of flexible filaments on the terminal disc of the egg processes, lower mean of egg bare diameter and egg full diameter (82.9 and 96.3  $\mu\text{m}$ , respectively, in the new species vs. 95.0 and 109.7  $\mu\text{m}$ , respectively, in *Mac. papei*), smaller mean terminal disc width (4.1  $\mu\text{m}$  in the new species vs. 4.3  $\mu\text{m}$  in *Mac. papei*), and smaller mean inter processes distance (2.1  $\mu\text{m}$  in the new species vs. 4.3  $\mu\text{m}$  in *Mac. papei*).

***Mac. paulinae* Stec, Smolak, Kaczmarek and Michalczyk, 2015** known only from type locality in Kenya [16], by lack of dorso-lateral patches of granulation, smaller maximum size of cuticular pores (up to 1.8  $\mu\text{m}$  in the new species vs. up to 0.5  $\mu\text{m}$  in *Mac. paulinae*), different oral cavity armature (*hufelandi* type in the new species vs. *maculatus* type in *Mac. paulinae*), different third band of teeth (three dorsal and three ventral teeth in the new species vs. single dorsal and single ventral tooth in *Mac. paulinae*), lunules I–III with hardly visible teeth, larger buccal tube external and internal width with higher *pt* (5.2–8.4 [13.5–17.0] and 3.4–5.6  $\mu\text{m}$  [8.7–11.9], respectively, in the new species vs. 2.2–4.6  $\mu\text{m}$  [8.8–12.6] and vs. 1.0–2.9  $\mu\text{m}$  [3.5–8.1], respectively, in *Mac. paulinae*), longer ventral lamina (19.9–32.9  $\mu\text{m}$  in the new species vs. 15.0–19.6  $\mu\text{m}$  in *Mac. paulinae*), longer macroplacoid row with higher *pt* (16.3–30.2  $\mu\text{m}$  [49.1–57.9] in the new species vs. 9.2–14.4  $\mu\text{m}$  [32.5–40.4] in *Mac. paulinae*), longer placoid row with higher *pt* (19.2–35.2  $\mu\text{m}$  [57.9–67.9] in new species vs. 10.8–17.4  $\mu\text{m}$  [37.8–48.9] in *Mac. paulinae*), larger egg bare and full diameter (74.9–86.9 and 87.8–103.9  $\mu\text{m}$ , respectively, in the new species vs. 57.0–70.5 and 66.3–85.6  $\mu\text{m}$ , respectively, in *Mac. paulinae*) and absence of filaments on egg processes discs.

***Mac. polypiformis* Roszkowska, Ostrowska, Stec, Janko and Kaczmarek, 2017** known only from type locality in Ecuador [38], by different oral cavity armature (*hufelandi* type in the new species vs. *maculatus* type in *Mac. polypiformis*), lunules I–III with hardly visible teeth, longer buccal tube (33.2–52.6  $\mu\text{m}$  in the new species vs. 24.4–32.5  $\mu\text{m}$  in *Mac. polypiformis*), stylet supports inserted in more caudal position with higher *pt* (26.2–43.0  $\mu\text{m}$

[78.0–82.8] in the new species vs. 17.1–23.5  $\mu\text{m}$  [70.1–72.9] in *Mac. polypiformis*), larger buccal tube external and internal width and with higher *pt* (5.2–8.4 [13.5–17.0] and 3.4–5.6  $\mu\text{m}$  [8.7–11.9], respectively, in the new species vs. 2.8–4.0 [11.0–13.0] and 1.6–2.4  $\mu\text{m}$  [6.1–8.6], respectively, in *Mac. polypiformis*), longer ventral lamina with larger *pt* (19.9–32.9  $\mu\text{m}$  [56.0–66.2] in the new species vs. 13.5–17.3  $\mu\text{m}$  [52.1–55.1] in *Mac. polypiformis*), longer macroplacoid 1 (8.2–16.0  $\mu\text{m}$  in the new species vs. 5.2–6.8  $\mu\text{m}$  in *Mac. polypiformis*), longer macroplacoid 2 with higher *pt* (6.0–11.2  $\mu\text{m}$  [17.2–22.0] in the new species vs. 2.8–4.1  $\mu\text{m}$  [11.4–14.5] in *Mac. polypiformis*), longer microplacoid (2.4–4.9  $\mu\text{m}$  in the new species vs. 1.5–2.3  $\mu\text{m}$  in *Mac. polypiformis*), longer macroplacoid row with higher *pt* (16.3–30.2  $\mu\text{m}$  [49.1–57.9] in the new species vs. 9.0–11.8  $\mu\text{m}$  [34.3–39.9] in *Mac. polypiformis*) and longer placoid row with higher *pt* (19.2–35.2  $\mu\text{m}$  [57.9–67.9] in the new species vs. 11.1–14.5  $\mu\text{m}$  [41.4–49.0] in *Mac. polypiformis*), larger egg bare and full diameter (74.9–86.9 and 87.8–103.9  $\mu\text{m}$ , respectively, in the new species vs. 61.9–70.5 and 70.4–81.2  $\mu\text{m}$ , respectively, in *Mac. polypiformis*) and absence of filaments on egg processes discs.

*Mac. porifini* Kuzdrowska, Mioduchowska, Gawalak, Bartylak, Kepel, Kepel and Kaczmarek 2021 known only from the type locality in Madagascar [39] by presence of eyes, different oral cavity armature (*hufelandi* type in the new species vs. *patagonicus* type in *Mac. porifini*), lunules I–III with hardly visible teeth, presence of dentate lunules on legs IV, higher *pt* of macroplacoid row (49.1–57.9 in the new species vs. 36.2–47.7 in *Mac. porifini*), higher *pt* of placoid row (57.9–67.9 in the new species vs. 43.5–57.7 in *Mac. porifini*), larger egg bare diameter (74.9–86.9  $\mu\text{m}$  in the new species vs. 72.2–74.0  $\mu\text{m}$  in *Mac. porifini*), absence of very small irregular granules on the surface of the discs and absence of micro granulation on the teeth of the terminal discs of egg process.

#### Genetic variability

Aligned sequences (obtained in our study and downloaded from GenBank) were trimmed to 609, 554, 682 and 200 bp for COI (21 sequences selected from GenBank—one sequence per species; (see Supplementary Materials—SM2)), 18S rRNA (20 sequences selected from GenBank—one sequence per species; see Supplementary Materials—SM3), 28S rRNA (16 sequences selected from GenBank—one sequence per species; see Supplementary Materials—SM4) and ITS2 (12 sequences selected from GenBank one sequence per species; see Supplementary Materials—SM5) molecular markers, respectively.

The analysis of the p-distances between our COI sequence (GenBank accession number: MW656266) and sequences of species from the genus *Macrobiotus* ranged from the most similar 17% for *Mac. canaricus* (GenBank accession number: MH063925 [67]), to least similar 25% for *Mac. kristenseni* Guidetti, Peluffo, Rocha, Cesari and Moly de Peluffo [74] (GenBank accession number: KC193575 [74]). In the conservative 18S rRNA gene fragment we observed no differences between our sequence (GenBank accession number: MW680641) and sequences of *Mac. hannaë* (GenBank accession number: HQ604975 [75]). In turn, the uncorrected genetic p-distances between the least similar *Mac. polonicus* Pilato, Kaczmarek, Michalczyk and Lisi [37] (GenBank accession number: HM187580 [76]) was 4%. The analysis of the p-distances between our sequence of 28S rRNA (GenBank accession number: MW680644) and similar sequences of the genus *Macrobiotus* are as follows: the most similar was *Mac. hannaë* (GenBank accession number: MH063924, Nowak and Stec [68]) with p-distance of 1% and the least similar was *Mac. polypiformis* (GenBank accession number: KX810009 [38]) and *Mac. scoticus* Stec, Morek, Gasiorek, Blagden and Michalczyk [77] (GenBank accession number: KY797266 [68]) with p-distance of 10%. In turn, the ranges of p-distances between our ITS-2 sequence (GenBank accession number: MW680418) and the most similar *Mac. hannaë* (GenBank accession number: MH063923 [68]) was 11% and the least similar was *Mac. polypiformis* (GenBank accession number: KX810010 [38])—33%.

**Genus:** *Mesobiotus* Vecchi, Cesari, Bertolani, Jönsson, Rebecchi and Guidetti, 2016 [78]

*Mesobiotus skorackii* Kaczmarek, Zawierucha, Buda, Stec, Gawlak, Michalczyk and Roszkowska, 2018 [15]

(Tables 6 and 7)

**Table 6.** Measurements (in  $\mu\text{m}$ ) and *pt* values of selected morphological structures of individuals of *Mesobiotus skorackii* mounted in Hoyer's medium (N—number of specimens/structures measured; RANGE refers to the smallest and the largest structure among all measured specimens; SD—standard deviation; *pt*—ratio of the length of a given structure to the length of the buccal tube expressed as a percentage).

Character	N	Range				Mean		SD			
		$\mu\text{m}$	$\mu\text{m}$	$\mu\text{m}$	$\mu\text{m}$	<i>pt</i>	$\mu\text{m}$	<i>pt</i>			
Body length	11	281	–	485	818	–	1102	418	991	62	76
<b>Buccopharyngeal tube</b>											
Buccal tube length	11	34.3	–	47.6		–		42.0	–	4.4	–
Stylet support insertion point	11	25.9	–	35.6	74.6	–	77.0	31.7	75.5	3.2	0.8
Buccal tube external width	11	5.9	–	8.0	15.2	–	18.0	7.0	16.8	0.7	0.7
Buccal tube internal width	11	3.8	–	5.6	11.0	–	12.7	4.9	11.5	0.6	0.6
Ventral lamina length	11	21.0	–	29.8	54.4	–	62.9	25.5	60.6	2.9	2.7
<b>Placoid lengths</b>											
Macroplacoid 1	11	4.3	–	6.7	11.6	–	14.3	5.5	13.1	0.7	1.0
Macroplacoid 2	11	3.7	–	5.4	9.8	–	12.6	4.7	11.3	0.5	0.8
Macroplacoid 3	11	4.1	–	6.4	11.1	–	13.8	5.3	12.5	0.7	0.9
Microplacoid	11	2.5	–	4.8	6.7	–	10.3	3.6	8.5	0.6	1.2
Macroplacoid row	11	15.3	–	22.9	44.4	–	48.2	19.4	46.2	2.4	1.2
Placoid row	11	19.7	–	27.6	54.2	–	60.5	24.4	57.9	2.9	1.7
<b>Claw 1 heights</b>											
External primary branch	11	6.9	–	11.2	18.3	–	26.2	9.5	22.5	1.4	2.1
External secondary branch	11	5.6	–	9.5	14.5	–	20.7	7.1	16.9	1.3	2.1
Internal primary branch	11	6.8	–	11.4	19.8	–	25.9	9.5	22.6	1.3	1.7
Internal secondary branch	11	5.3	–	8.9	15.4	–	20.6	7.7	18.2	1.0	1.5
<b>Claw 2 heights</b>											
External primary branch	11	6.9	–	11.4	20.2	–	26.7	9.9	23.6	1.4	1.8
External secondary branch	11	5.4	–	9.7	15.8	–	22.7	8.0	19.0	1.6	2.6
Internal primary branch	11	7.1	–	11.0	20.1	–	25.6	9.2	21.9	1.0	1.6
Internal secondary branch	10	5.5	–	10.3	16.0	–	24.2	7.6	17.9	1.3	2.6
<b>Claw 3 heights</b>											
External primary branch	11	7.8	–	11.6	22.8	–	28.1	10.4	24.7	1.2	1.8
External secondary branch	11	5.2	–	9.9	15.2	–	23.1	8.2	19.4	1.3	2.7
Internal primary branch	11	6.7	–	10.7	19.6	–	27.2	9.5	22.6	1.2	1.9
Internal secondary branch	11	5.3	–	9.6	15.5	–	22.2	8.0	18.9	1.1	2.0
<b>Claw 4 lengths</b>											
Anterior primary branch	11	7.2	–	13.0	21.0	–	29.6	11.4	27.0	1.7	2.4
Anterior secondary branch	11	5.9	–	10.3	17.1	–	23.7	9.0	21.3	1.3	2.1
Posterior primary branch	11	6.5	–	13.2	18.9	–	30.7	10.7	25.3	2.1	3.6
Posterior secondary branch	11	5.2	–	10.7	15.1	–	24.4	8.5	20.1	1.5	2.6

**Table 7.** Measurements (in  $\mu\text{m}$ ) of selected morphological structures of eggs of *Mesobiotus skorackii* mounted in Hoyer's medium (N—number of specimens/structures measured; RANGE refers to the smallest and the largest structure among all measured eggs; SD—standard deviation).

Character	N	Range	Mean	SD
Egg bare diameter	16	62.8–89.5	74.1	8.1
Egg full diameter	16	87.6–113.6	102.2	7.6
Process height	48	13.5–20.4	17.1	1.7
Process base width	48	12.7–19.0	16.1	1.4
Process base/height ratio	48	72–127%	95%	12%
Inter-process distance	48	1.3–5.6	3.5	1.2
Number of processes on the egg circumference	16	9–13	11.1	1.2

*Locality:* 51°24'21" N, 116°14'27" W, 1900 m asl, Canada, Alberta, Banff National Park, near east end of the Louise Lake, moss on stone, May 2019, leg. Milena Roszkowska and Łukasz Kaczmarek.

**Material examined:** The 63 specimens; 44 animals and 19 eggs were mounted on microscope slides in Hoyer's medium, four eggs and four animals prepared for SEM and six animals prepared for molecular analyses. However, DNA sequences were obtained from one female specimen (exoskeleton) which was later mounted on microscope slide in Hoyer's medium and included into type series.

**Depositories:** All specimens (slides: CN8.\*, where the asterisk can be substituted by any of the following numbers: 5–7, 31–40, 42–43, 45, 115/S) are deposited at the Department of Animal Taxonomy and Ecology, Institute of Environmental Biology, Adam Mickiewicz University in Poznań, Uniwersytetu Poznańskiego 6, 61-614 Poznań, Poland.

**Short diagnosis:**

Adults (measurements and statistics in Table 6). Body white in living animals and transparent after fixation in Hoyer's medium, eyes present, cuticle smooth. Bucco-pharyngeal apparatus of the *Macrobotus* type, with ventral lamina and ten peribuccal lamellae. Mouth antero-ventral. Oral cavity armature of the *harmsworthi* type. Pharyngeal bulb spherical with triangular apophyses, three rod-shaped macroplacoids and a triangular microplacoid. Macroplacoid length sequence  $2 < 3 < 1$ . The first macroplacoid narrower anteriorly, the second without constrictions and the third with a small, subterminal constriction. Claws of the *Mesobotus* type. Lunules under claws I–III smooth and slightly dentated under claws IV. Thin cuticular bars under claws I–III present. Granulation hardly visible on legs I–III, whereas on legs IV always clearly marked.

Eggs (measurements and statistics in Table 7). Eggs laid freely, white and spherical. Egg processes in the shape of short and wide sharpened cones. Egg processes reticulated and surrounded by six areolae delimited by thin brims which are often discontinuous, thus areolae are not always fully formed (semi-areolation). Surface inside the areolae with clearly visible wrinkles.

**DNA sequences**

We obtained good quality sequences for the applied molecular markers:

**COI:** single sequence; 631 bp long;

**18S rRNA:** two sequences; 667–715 bp long;

**28S rRNA:** single sequence; 735 bp long.

**Genetic variability**

Aligned sequences (obtained in our study and downloaded from GenBank) were trimmed to 565, 474 and 713 bp for COI (14 sequences, selected from GenBank—one sequence per species; (see Supplementary Materials—SM6)), 18S rRNA (two sequences selected from GenBank—one sequence per species; see below) and 28S rRNA (13 sequences selected from GenBank—one sequence per species) molecular markers, respectively.

The ranges of uncorrected genetic p-distances between obtained COI sequence of *Meb. skorackii* (GenBank accession number: MW656257) and species of the genus *Mesobotus*, for which sequences are available from GenBank, are as follows: 20–26%, with the most similar being *Meb. cf. barabanovi* (GenBank accession number: MN313170 [23]) and *Meb. occultatus* Kaczmarek, Zawierucha, Buda, Stec, Gawlak, Michalczyk and Roszkowska, [15] (GenBank accession number: MH195152 [15]) and the least similar being *Meb. dilimanensis* Itang, Stec, Mapalo, Mirano-Bascos and Michalczyk [79] (GenBank accession number: MN257047 [79]). In case 18S rRNA only two sequences were compared (because other 24 sequences deposited in GenBank were amplified using different sets of primers) and genetic p-distances between *Meb. harmsworthi* (at present undefined *Mesobotus* species; GenBank accession number: MH079462 [66]) and *Meb. philippinicus* Mapalo, Stec, Mirano-Bascos and Michalczyk [80] (GenBank accession number: KX129793 [80]) was 0.01%. In turn, the ranges of p-distances between obtained 28S rRNA sequence (GenBank accession number: MW680636) and sequences downloaded from GenBank was: 5–13%, with the most similar being *Meb. cf. barabanovi* (GenBank accession number: MN310388 [23]) as well as *Meb. harmsworthi* (at present undefined *Mesobotus* species; GenBank accession number: MH197264 [66]) and the least similar being *Meb. dilimanensis* (GenBank accession number: MN257049 [79]) (see Supplementary Materials—SM7).

#### 4. Discussion

Out of 10 provinces and three territories of Canada, limno-terrestrial tardigrades have been reported in eight provinces and two territories. Up to now, no tardigrades have been reported from Northwest Territories, Nova Scotia nor Prince Edward Island. The highest number of tardigrade species were recorded from Nunavut (70) and the lowest Manitoba (only one). Moreover, 18 species were recorded from Alberta, 55 from British Columbia, 33 from New Brunswick, 29 from Newfoundland and Labrador, 12 from Ontario, 13 from Quebec, 3 from Saskatchewan and 5 from Yukon [13,14]. Only one species of the genus *Bryodelphax*, i.e., *Bry. parvulus* has been recorded from British Columbia and Nunavut. In case of the genus *Macrobiotus*, four species i.e., *Mac. echinogenitus* Richters [81], *Mac. hufelandi*, *Mac. occidentalis* Murray [82] and *Mac. virgatus* Murray [82] were recorded from Alberta, British Columbia, New Brunswick, Newfoundland and Labrador, Nunavut, Ontario and Quebec. Among these, only *Mac. hufelandi* belongs to the *hufelandi* group. Three species of the genus *Mesobiotus*, i.e., *Meb. harmsworthi* (Murray [83]), *Meb. montanus* (Murray [82]) and *Meb. pilatoi* (Binda and Rebecchi [84]) were recorded from Alberta, British Columbia, New Brunswick, Newfoundland and Labrador, Nunavut, Ontario and Quebec [14]. What is more intriguing, some species reported from Canada in the past are now considered as group of species or species with problematic taxonomical status [14].

Summarizing, from Canada only 121 tardigrade species and subspecies are known. Taking into consideration the area of the country (ca. 10 million km<sup>2</sup>) and its diversity such as habitats and ecosystem, it is obvious that this number is highly underestimated. For comparison, USA, with the similar country area, has more than 220 species reported [14]. This contrast is even more spectacular while comparing Canadian tardigrade fauna with the number of tardigrade species from much smaller areas like e.g., Costa Rica (ca. 51,000 km<sup>2</sup> and 63 species known), Finland (ca. 340,000 km<sup>2</sup> and 68 species known), Italy (ca. 300,000 km<sup>2</sup> and 234 species known) or Poland (ca. 312,000 km<sup>2</sup> and 111 species known) [68,85–89]. The number of tardigrade species from Canada is expected to be much higher than reported up to date, especially that in the present study, in one analyzed sample, we found two species new for science and one new record for the country.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/article/10.3390/d13080394/s1>, SM1—Estimates of evolutionary divergence between 28S rRNA sequences of *Bryodelphax mareki* sp. nov based on p-distances, SM2—Estimates of evolutionary divergence between COI sequences of *Macrobiotus birendrai* sp. nov based on p-distances, SM3—Estimates of evolutionary divergence between 18S rRNA sequences of *Macrobiotus birendrai* sp. nov based on p-distances, SM4—Estimates of evolutionary divergence between 28S rRNA sequences of *Macrobiotus birendrai* sp. nov based on p-distances, SM5—Estimates of evolutionary divergence between ITS-2 sequences of *Macrobiotus birendrai* sp. nov based on p-distances, SM6—Estimates of evolutionary divergence between COI sequences of *Mesobiotus skorackii* based on p-distances, SM7—Estimates of evolutionary divergence between 28S rRNA sequences of *Mesobiotus skorackii* based on p-distances [13,15–17,23,30,38,39,41,60,67,70,73–80,90–103].

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