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

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 macroplacoids and microplacoid, 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, Zarriierucha, Buda, Stec, Gawlak, Michalczysk 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
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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 Gaśiorek 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 Dęgma [32], Bry. ol- szanowskii Kaczmarek, Parnikoza, Gawlak, Esefeld, Peter, Kozeretska and Roszkowska [33], Bry. parvuspolaris Kaczmarek, Zawierucha, Smykla and Michalczyk [20], Mac. dulciporus 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, Didusko 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 Sablea [40] and Mac. wandae Kayastha, Berdi, Mioduchowska, Gawlak, Łukasiewicz, Goldyn 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®® 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 | GGTCAACAAATCATAAGATATTGG TAACTTCAGGGTGACCAAAAAATCA | Folmer et al. [45] |
| 18S rRNA     | SSU01_F/forward SSU82_R/reverse | AACCTGGTTGATCCTGCCAGT TGATCCCTTCGACCGTTACCTAC | Sands et al. [46] |
| 28S rRNA     | 28SF0001/forward 28SR0990/reverse | ACCCvCynAATTTAAGCATAT CCTTGGTCCGGTGTTCAAGAC | Mironov et al. [47] |
| ITS-2        | ITS3/forward ITS4/reverse | GCATCGATGAAGAAGCACGC TCCTCCGCTTTATTATGC | 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 |
| *Bryodelphax mareki* 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 |
| *Macrobiotus birendrai* sp. nov. | MW656266 (CN8.101/S) | MW680641 (CN8.101/S) | MW680644 (CN8.101/S) | MW680418 (CN8.101/S) |
| *Mesobiotus skorackii* | 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)
Table 3. Measurements (in µm) 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 |
|----------------------------------------|-----|-------------|-------|-----|----------|
|                                        |     | µm          | sc    | µm  | sc       | µm  | sc    |
| Body length                            | 16  | 122 – 181   | 577 – 780 | 141 | 689 – 1560 | 146 | 749 |
| Scapular plate length                  | 16  | 18.4 – 24.0 | –      | 20.4 – 1.7 | – 19.5 |
| Head appendages lengths                |     |             |       |     |          |     |      |
| *Cirrus internus*                      | 16  | 5.3 – 7.3   | 26.8 – 34.6 | 6.2 | 30.6 – 0.5 | 2.7 | 5.3 |
| Cephalic papilla                       | 15  | 4.1 – 5.9   | 21.7 – 29.1 | 4.9 | 24.1 – 0.5 | 2.3 | 4.3 |
| *Cirrus externus*                      | 16  | 11.1 – 13.1 | 53.4 – 63.0 | 11.9 | 58.7 – 0.7 | 3.2 | 11.1 |
| Clava                                  | 12  | 4.3 – 6.2   | 20.4 – 33.0 | 5.2 | 26.1 – 0.5 | 3.7 | 4.8 |
| *Cirrus A*                             | 15  | 35.2 – 46.7 | 174.1 – 212.9 | 193.6 | 3.2 | 10.7 | 36.5 |
| *Cirrus A/body length ratio*           | 15  | 25% – 32%   | –      | 28% – 2% | – 25% |
| *Cirrus int/ext 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 |
| Claw 1 heights                         |     |             |       |     |          |     |      |
| Branch                                 | 16  | 5.8 – 8.4   | 30.3 – 42.0 | 7.6 | 37.5 – 0.7 | 2.8 | 7.0 |
| Spur                                   | 13  | 1.3 – 1.7   | 5.4 – 8.2 | 1.5 | 7.2 – 0.1 | 0.8 | 1.4 |
| 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 |
| Spur                                   | 14  | 1.0 – 1.5   | 5.2 – 7.2 | 1.3 | 6.6 – 0.1 | 0.7 | 1.4 |
| 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 |
| Spur                                   | 12  | 1.2 – 1.5   | 5.4 – 7.5 | 1.4 | 6.6 – 0.1 | 0.8 | 1.3 |
| 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 |
| Spur                                   | 13  | 1.4 – 1.9   | 6.3 – 8.9 | 1.6 | 7.8 – 0.1 | 0.7 | 1.6 |
| 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 μm.
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 μm.
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 μm.

**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 µm², \( \bar{x} = 7.5, N = 10 \)); on caudal plate (0.6–1.0 µm in diameter; 0–19 pores/100 µm², \( \bar{x} = 4.44, N = 10 \)) and on other plates (0.3–0.8 µm in diameter; 1–16 pores/100 µm², \( \bar{x} = 6.3, N = 10 \)). 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 weglerae group. Within this group, only *Bry. amphoterus* (Durante Pasa and Maucci [58]), *Bry. maculatus* Gašiorek, Stec, Morek, Marnissi and Michalczyk [58] and *Bry. nigripunctatus* Degma, Gašiorek, 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 (Gašiorek 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 (Gašiorek 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: Macrobiotoidea Thulin, 1928 [8]
Family: Macrobiotidae Thulin, 1928 [8]
Genus: Macrobiotus C.A.S. Schultze, 1834 [11]
Macrobiotus birendrai sp. nov.

*(Table 4, Figures 5–8)*

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

| Character | N | Range | Mean | SD | Holotype |
|-----------|---|-------|------|----|----------|
| Body length | 15 | 271 – 589 | 815 – 1291 | 449 | 133 | 368 | 978 |
| Buccopharyngeal tube | | | | | | | |
| 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 |
| Placoid lengths | | | | | | | |
| 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 |
| Claw 1 heights | | | | | | | |
| 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 |
|----------------------------|----|---------------------|------|-----|----------|
|                            |    | µm      | pt  | µm  | pt  | µm  | pt  | µm  | pt  |
| Claw 2 heights             |    |          |     |     |      |     |     |     |     |
| 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|
| Claw 3 heights             |    |          |     |     |      |     |     |     |     |
| 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|
| Claw 4 lengths             |    |          |     |     |      |     |     |     |     |
| 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 µm) 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 µm.
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 µm.
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 $\mu$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 Macrobiotus 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. hannae Nowak and Stec [68], Mac. crustulus Stec et al. [12], Mac. joannae Pilato and Binda [69], Mac. kamilae Coughlan and Stec [70], Mac. madagassus 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. polypliformis 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 μm in the new species vs. 2.1–3.3 μm 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 μm in the new species vs. 0.4–0.7 μm 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 μm in the new species vs. 1.6–2.7 μm 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. hannae 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 μm in the new species vs. up to 0.55 μm in Mac. hannae), smaller distance between egg processes (1.0–3.3 μm in the new species vs. 4.0–8.1 μm in Mac. hannae), smaller egg bare diameter (74.9–86.9 μm in the new species vs. 88.6–109.2 μm in Mac. hannae) 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 μm, respectively, in the new species vs. 19.3 and 6.4 μm, respectively, in Mac. joannae in specimen of body length 400 μm), smaller size of claws I and II external and internal primary branch (l: 8.5–13.2 and 8.2–12.7 μm, respectively; II: 8.1–14.0 and 8.6–13.6 μm, respectively, in the new species vs. I: 25.0 and 23.5 μm, respectively; II: 27.0 and 24.5 μm, respectively in Mac. joannae in specimen of body length 400 μm) and smaller size of claw IV anterior and posterior primary branch (9.5–15.8 and 8.4–14.2 μm, respectively in the new species vs. 28.0 and 27.0 μm, respectively, in Mac. joannae in specimen of body length 400 μm).

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 μm in the new species vs. up to 30.0 μm in Mac. madegassus), larger buccal tube external width (5.2–8.4 μm in the new species vs. up to 2.1 μm 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 μm), longer macroplacoids (l: 8.2–16.0 μm and II: 6.0–11.2 μm in the new species vs. I: up to 6.4 μm and II: up to 3.6 μm in Mac. madegassus in specimen of body length 316 μm), stylet supports inserted in more caudal position (26.2–43.0 μm in the new species vs. up to 20.4 μm in Mac. madegassus in specimen of body length 316 μm), 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 µm) 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 µm, respectively, in the new species vs. 118.5–123.5 and 100.6–105.7 µm, 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 µm in the new species vs. up to 0.8 µm 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 µm, respectively, in the new species vs. 70.7 and 82.1 µm, respectively, in Mac. noongaris), larger mean process height, process base width and terminal disc width (7.9, 6.6 and 4.1 µm, respectively, in the new species vs. 6.2, 5.0 and 3.3 µm, respectively, in Mac. noongaris), and smaller mean inter processes distance (2.1 µm in the new species vs. 3.4 µm 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 µm, respectively, in the new species vs. 95.0 and 109.7 µm, respectively, in Mac. papei), smaller mean terminal disc width (4.1 µm in the new species vs. 4.3 µm in Mac. papei), and smaller mean inter processes distance (2.1 µm in the new species vs. 4.3 µm 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 µm in the new species vs. up to 0.5 µm 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 µm [8.7–11.9], respectively, in the new species vs. 2.2–4.6 µm [8.8–12.6] and vs. 1.0–2.9 µm [3.5–8.1], respectively, in Mac. paulinae), longer ventral lamina (19.9–32.9 µm in the new species vs. 15.0–19.6 µm in Mac. paulinae), longer macroplacoid row with higher pt (16.3–30.2 µm [49.1–57.9] in the new species vs. 9.2–14.4 µm [32.5–40.4] in Mac. paulinae), longer placoid row with higher pt (19.2–35.2 µm [57.9–67.9] in new species vs. 10.8–17.4 µm [37.8–48.9] in Mac. paulinae), larger egg bare and full diameter (74.9–86.9 and 87.8–103.9 µm, respectively, in the new species vs. 57.0–70.5 and 66.3–85.6 µm, 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 µm in the new species vs. 24.4–32.5 µm in Mac. polypiformis), stylet supports inserted in more caudal position with higher pt (26.2–43.0 µm.
[78.0–82.8] in the new species vs. 17.1–23.5 µm [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 µm [8.7–11.9], respectively, in the new species vs. 2.8–4.0 [11.0–13.0] and 1.6–2.4 µm [6.1–8.6], respectively, in Mac. polypiformis), longer ventral lamina with larger pt (19.9–32.9 µm [56.0–66.2] in the new species vs. 13.5–17.3 µm [52.1–55.1] in Mac. polypiformis), longer macroplacoid 1 (8.2–16.0 µm in the new species vs. 5.2–6.8 µm in Mac. polypiformis), longer macroplacoid 2 with higher pt (6.0–11.2 µm [17.2–22.0] in the new species vs. 2.8–4.1 µm [11.4–14.5] in Mac. polypiformis), longer microplacoid (2.4–4.9 µm in the new species vs. 1.5–2.3 µm in Mac. polypiformis), longer macroplacoid row with higher pt (16.3–30.2 µm [49.1–57.9] in the new species vs. 9.0–11.8 µm [34.3–39.9] in Mac. polypiformis) and longer placoid row with higher pt (19.2–35.2 µm [57.9–67.9] in the new species vs. 11.1–14.5 µm [41.4–49.0] in Mac. polypiformis), larger egg bare and full diameter (74.9–86.9 and 87.8–103.9 µm, respectively, in the new species vs. 61.9–70.5 and 70.4–81.2 µm, respectively, in Mac. polypiformis) and absence of filaments on egg processes disc.

Mac. porifini Kuzdrowska, Mioduchowska, Gawalak, Bartyłak, 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 µm in the new species vs. 72.2–74.0 µm 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. canarius (GenBank accession number: MH063925 [67]), to least similar 25% for Mac. kristensi 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. hannae (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. hannae (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. hannae (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 µm) 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  |
|----------------------------------|----|-----------|--------|-----|
|                                  |    | µm        | pt     | µm  | pt  | µm    | pt    |
| Body length                      | 11 | 281 – 485 | 1102   | 418 | 991 | 62    | 76    |
| Buccopharyngeal tube             |    |           |        |     |     |       |       |
| Buccal tube length               | 11 | 34.3 – 47.6| 42.0   | 4.4 | 76  | 62    | 2.7   |
| Stylet support insertion point   | 11 | 25.9 – 35.6| 31.7   | 5.3 | 12  | 6.6   | 0.8   |
| Buccal tube external width       | 11 | 5.9 – 8.0  | 7.0    | 3.6 | 8.5 | 0.6   | 0.7   |
| Buccal tube internal width       | 11 | 3.8 – 5.6  | 4.9    | 3.0 | 11.5 |       |       |
| Ventral lamina length            | 11 | 21.0 – 29.8| 25.5   | 2.9 | 60.6| 2.7   | 2.7   |
| Placoid lengths                  |    |           |        |     |     |       |       |
| Macroplacoid 1                   | 11 | 4.3 – 6.7  | 5.5    | 0.7 | 13.1 |       |       |
| Macroplacoid 2                   | 11 | 3.7 – 5.4  | 4.7    | 0.5 | 11.3 |       |       |
| Macroplacoid 3                   | 11 | 4.1 – 6.4  | 5.3    | 0.7 | 12.5 |       |       |
| Microplacoid                     | 11 | 2.5 – 4.8  | 3.6    | 0.6 | 8.5  | 0.6   | 0.7   |
| Macroploid row                   | 11 | 15.3 – 22.9| 19.4   | 2.4 | 46.2 | 2.4   | 1.4   |
| Placoid row                      | 11 | 19.7 – 27.6| 24.4   | 2.9 | 57.9 | 1.7   | 1.7   |
| Claw 1 heights                   |    |           |        |     |     |       |       |
| External primary branch          | 11 | 6.9 – 11.2| 26.2   | 9.5 | 22.5 | 1.6   | 1.4   |
| External secondary branch        | 11 | 5.6 – 9.5 | 20.7   | 7.1 | 19.0 | 1.6   | 1.7   |
| Internal primary branch          | 11 | 6.8 – 11.4| 25.9   | 9.5 | 22.6 | 1.7   | 1.5   |
| Internal secondary branch        | 11 | 5.3 – 8.9 | 20.6   | 7.7 | 18.2 | 1.0   | 1.7   |
| Claw 2 heights                   |    |           |        |     |     |       |       |
| External primary branch          | 11 | 6.9 – 11.2| 26.7   | 9.9 | 23.6 | 1.8   | 1.4   |
| External secondary branch        | 11 | 5.4 – 9.7 | 22.7   | 8.0 | 19.0 | 1.6   | 1.6   |
| Internal primary branch          | 11 | 7.1 – 11.0| 25.6   | 9.2 | 21.9 | 1.0   | 1.6   |
| Internal secondary branch        | 10 | 5.5 – 10.3| 24.2   | 7.6 | 17.9 | 1.3   | 1.5   |
| Claw 3 heights                   |    |           |        |     |     |       |       |
| External primary branch          | 11 | 7.8 – 11.6| 28.1   | 10.4| 24.7 | 1.2   | 1.8   |
| External secondary branch        | 11 | 5.2 – 9.9 | 23.1   | 8.2 | 19.4 | 1.3   | 2.7   |
| Internal primary branch          | 11 | 6.7 – 10.7| 27.2   | 9.5 | 22.6 | 1.2   | 1.9   |
| Internal secondary branch        | 11 | 5.3 – 9.6 | 22.2   | 8.0 | 18.9 | 1.1   | 2.0   |
| Claw 4 lengths                   |    |           |        |     |     |       |       |
| Anterior primary branch          | 11 | 7.2 – 13.0| 29.6   | 11.4| 27.0 | 1.7   | 2.4   |
| Anterior secondary branch        | 11 | 5.9 – 10.3| 23.7   | 9.0 | 21.3 | 1.3   | 2.1   |
| Posterior primary branch         | 11 | 6.5 – 13.2| 30.7   | 10.7| 25.3 | 2.1   | 3.6   |
| Posterior secondary branch       | 11 | 5.2 – 10.7| 24.4   | 8.5 | 20.1 | 1.5   | 2.6   |

Table 7. Measurements (in µm) 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  |
|----------------------------------|----|-----------|--------|-----|
|                                  |    | µm        | pt     | µm  | pt  | µm    | pt    |
| 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 *Macrobiotus* 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 *Mesobiotus* 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 *Mesobiotus*, 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. occulatus* 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 *Mesobiotus* 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 *Mesobiotus* 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 Mesobiatus, 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$^2$) 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$^2$ and 63 species known), Finland (ca. 340,000 km$^2$ and 68 species known), Italy (ca. 300,000 km$^2$ and 234 species known) or Poland (ca. 312,000 km$^2$ 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 Mesobiatus skorackii based on p-distances, SM7—Estimates of evolutionary divergence between 28S rRNA sequences of Mesobiatus skorackii based on p-distances [13,15–17,23,30,38,39,41,60,67,70,73–80,90–103].

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