



Research article

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***Macrobiotus polypiformis* sp. nov., a new tardigrade (Macrobiotidae; *hufelandi* group) from the Ecuadorian Pacific coast, with remarks on the claw abnormalities in eutardigrades**

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Abstract. From a moss sample collected in the Manabí Province in Ecuador, we extracted 96 specimens of a new species of eutardigrade. No eggs were found. In order to obtain eggs, an *in vitro* culture was prepared. In total, 136 specimens (including ten simplex), one exuvia and 44 eggs (including two with embryos) of the new species were obtained. In addition to the traditional taxonomic description with morphometrics, light and scanning microscopy imaging, we also provide nucleotide sequences of three nuclear (18S rRNA, 28S rRNA, ITS-2) and one mitochondrial (COI) DNA fragments of the new species. *Macrobiotus polypiformis* sp. nov. belongs to the *hufelandi* group and is most similar to *Ma. paulinae* Stec, Smolak, Kaczmarek & Michalczyk, 2015, but differs from it mainly by the lack of dorso-lateral patches of granulation on the cuticle, egg processes with longer and more numerous filaments and in some morphometric characters of both eggs and adults. Moreover, we provide a short discussion on the modifications/abnormalities of the claws in eutardigrades and the possible consequences on the taxonomic status of *Mesobiotus armatus* (Pilato & Binda, 1996), suggesting its consideration as *species inquirenda* (with uncertain taxonomic status).

Keywords. DNA barcoding, Eutardigrada, new species, Neotropic, water bears.

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Introduction

Ecuador, located in the north-western part of South America, is divided into three main regions: the western coastal lowlands, the Andean mountain range and the eastern Amazon Basin (the Oriente) (St Louis *et al.* 2009). Ecuador also owns the Galápagos Islands, situated about 1,000 km west of the mainland.

Manabí is one of the Ecuadorian provinces located in the coastal region. In terms of area, it is the largest province on the coast and the fifth in the country. The climate there is determined by the Humboldt Current and the weather phenomenon El Niño, and oscillates from subtropical and dry on the coast to wet and extremely humid further inland.

The Phylum Tardigrada consists of over 1,200 species (Guidetti & Bertolani 2005; Degma & Guidetti 2007; Degma *et al.* 2009–2016; Vicente & Bertolani 2013), inhabiting terrestrial, freshwater and marine environments throughout the world (Ramazzotti & Maucci 1983; McInnes 1994; Nelson *et al.* 2015).

Tardigrades of South America are very poorly known and, until now, only 227 taxa have been reported from this region (ca 18% of all known species) (Kaczmarek *et al.* 2015 and literature cited therein; Londoño *et al.* 2015; Roszkowska *et al.* 2015, 2016). Water bears are known best from Argentina, Chile and Brazil with 119, 63 and 61 taxa, respectively. In comparison, the Ecuadorian tardigrade fauna is almost unknown, with only 21 species reported from this region to date (Kaczmarek *et al.* 2015 and literature cited therein). Among them, only one species of the *Macrobiotus hufelandi* group was recorded: *Ma. hibiscus* de Barros, 1942. However, this species has an uncertain taxonomic position and geographic distribution, and needs redescription (Kaczmarek *et al.* 2015).

The *Macrobiotus hufelandi* group is a cosmopolitan complex with 42 known species (Stec *et al.* 2015; Bąkowski *et al.* 2016). They are characterized by the presence of an oral cavity armature composed of three rows of teeth (sometimes visible only in SEM), two macroplacoids and a microplacoid in the pharynx, and eggs with processes in the shape of inverted goblets. The great majority of the *hufelandi* group species exhibit a restricted distribution (e.g., *Ma. iharosi* Pilato, Binda & Catanzaro, 1991, known from Democratic Republic of Congo, the Republic of South Africa and Tanzania; *Ma. madegassus* Maucci, 1993, known only from Madagascar; *Ma. patagonicus* Maucci, 1988 known only from Argentina and Chile) or are known only from their type localities (e.g., *Ma. anemone* Meyer, Domingue & Hilton, 2014, known only from Louisiana (USA); *Ma. kristenseni* Guidetti, Peluffo, Rocha, Cesari & Moly de Peluffo, 2013 or *Ma. kazmierskii* Kaczmarek & Michalczyk, 2009, known only from southern Argentina) (Kaczmarek *et al.* 2015, 2016).

In this paper we describe a new species of the *hufelandi* group, *Macrobiotus polypiformis* sp. nov., collected from the Ecuadorian Pacific coast, in Manabí Province. We have used an integrative approach (SEM observation and DNA sequencing, together with *in vitro* culturing) and as a result we were able to observe and describe details of egg morphology that would probably have been overlooked using solely traditional taxonomic tools. We also discuss the presence of modifications/abnormalities in tardigrade claws and their consequences for the taxonomic status of *Mesobiotus armatus* (Pilato & Binda, 1996).

Material and methods

Sample processing and culturing of specimens

The moss sample from a concrete wall was collected by Milena Roszkowska and Łukasz Kaczmarek on the 3rd of January 2015 in the Manabí Province, Ecuador. The sample was collected and examined for terrestrial tardigrades using standard methods (Dastyh 1980), with modifications described in Stec *et al.* (2015). After extraction, a total of 96 tardigrades and no eggs of the new species were obtained. Of these, 84 specimens were mounted on microscope slides in Hoyer's medium and the remaining 12, in order to obtain eggs, were placed in a plastic Petri dish containing "Żywiec Zdrój" spring water and unicellular freshwater algae (*Chlorococcum* sp. and *Chlorella* sp.). To aid tardigrade locomotion, the Petri dish bottom was scratched with fine sandpaper. The culture was maintained at room temperature (ca 22 °C) and out of direct sunlight. After a few weeks of culturing, 7 adults and 29 eggs were obtained. All were mounted on microscope slides in Hoyer's medium.

To enable Scanning Electron Microscope (SEM) observation and DNA sequencing, another sample extraction and culture was undertaken. From this second laboratory culture, 25 adults and 15 eggs were prepared for SEM analysis and eight individuals were used for DNA extraction and sequencing.

All measurements and photomicrographs were taken using an Olympus BX41 phase contrast microscope (PCM) associated with an ARTCAM-300Mi digital camera (Olympus Corporation, Shinjuku-ku, Japan) and Olympus BX63 differential interference contrast (DIC) associated with an Olympus XC10 camera (Olympus Corporation, Shinjuku-ku, Japan). In order to obtain clean and fully extended specimens for SEM, tardigrades and their eggs were prepared using methods described in Stec *et al.* (2015). Animals and eggs were examined under high vacuum in a Versa 3D DualBeam Scanning Electron Microscope at the ATOMIN facility of the Jagiellonian University, Kraków, Poland.

Sample size for morphometry was chosen following recommendations by Stec *et al.* (2016a). All measurements, made with the QuickPhoto Camera 2.3 software, are given in micrometres (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 the hind legs. The terminology used to describe buccal apparatus and more specifically the oral cavity armature, and used in differential diagnoses, follows Michalczyk & Kaczmarek (2003) and Guidetti *et al.* (2012). Buccal tube length and the level of the stylet support insertion point were measured according to Pilato (1981). The buccal tube width was measured as the external and internal diameter at the level of the stylet support insertion point. The macroplacoid length sequence is given according to Kaczmarek *et al.* (2014). Lengths of the claw branches were measured from the base of the claw to the top of the branch, excluding lunules, but including accessory points. The *pt* ratio is the ratio of the length of a given structure to the length of the buccal tube expressed as a percentage (Pilato 1981). The distance between egg processes was measured as the shortest line connecting the base edges of the two closest processes. Morphometric data were handled using the "Macrobiotidae" ver. 1.2 template available from the Tardigrada Register (Michalczyk & Kaczmarek 2013). Tardigrade taxonomy follows Bertolani *et al.* (2014).

Genotyping

Eight individuals were used for DNA isolation and sequencing. Each specimen was identified under LM before DNA extraction. The goal was to amplify four commonly used molecular markers: 18S rRNA, 28S rRNA, ITS-2, COI. The first three of these are nuclear whereas COI is a mitochondrial marker. These markers differ in mutation rate. The first two are conservative, while the last two are characterised by a relatively high mutation rate, making them suitable for different purposes. The 18S rRNA together with 28S rRNA are used for resolving relationships at higher taxonomic levels such as families and genera (e.g., Bertolani *et al.* 2014) whereas COI and ITS-2 are appropriate for examining intra and inter-

Table 1. Primers used for sequencing of four DNA fragments (one mitochondrial and three nuclear) of *Macrobiotus polypiformis* sp. nov.

DNA fragment	Primer name	Primer direction	Primer sequence (5'-3')	Source
18S rRNA	SSU01_F	forward	AACCTGGTTGATCCTGCCAGT	Sands <i>et al.</i> (2008)
	SSU82_R	reverse	TGATCCTTCTGCAGGTTACCTAC	Sands <i>et al.</i> (2008)
28S rRNA	28SF0001	forward	ACCCVCYNAATTTAAGCATAT	Mironov <i>et al.</i> (2012)
	28SR0990	reverse	CCTTGGTCCGTGTTTCAAGAC	Mironov <i>et al.</i> (2012)
COI	LCO1490	forward	GGTCAACAAATCATAAAGATATTGG	Folmer <i>et al.</i> (1994)
	HCO2198	reverse	TAAACTTCAGGGTGACCAAAAAATCA	Folmer <i>et al.</i> (1994)
ITS-2	ITS3	forward	GCATCGATGAAGAACGCAGC	White <i>et al.</i> (1990)
	ITS4	reverse	TCCTCCGCTTATTGATATGC	White <i>et al.</i> (1990)

specific genetic variation (e.g., Hebert *et al.* 2003; Cesari *et al.* 2009; Bertolani *et al.* 2011; Welnicz *et al.* 2011; Vicente & Bertolani 2013; Morek *et al.* 2016).

DNA extraction from single individuals was carried out according to the protocol by Casquet *et al.* (2012) with modification described in detail in Stec *et al.* (2015). The method comprises two major reagents; 75-150 µm wet bead Chelex® 100 resin (Bio-Rad) material chelating Mg²⁺ ions which are cofactors of DNases (DNA protection from degradation) and Proteinase K (Qiagen®) which digests proteins in cell membranes.

Amplification of each DNA fragment (PCR) was conducted in a total volume of 20.0 µl (see Table 1 for primers; for PCR cocktails and programs see Stec *et al.* 2015). Separation of PCR products were carried out by 1% agarose gel electrophoresis stained with Midori Green (Nippon Genetics) and purified with the NucleoSpin® Gel and PCR Clean-up kit and suspended in 20.0 µl of Tris/HCl (5 mM). Sequencing reactions were done in a total volume of 10.0 µl containing: 1.0 µl 5 × buffer, 1.0 µl BrightDye® Terminator Cycle Sequencing Kit v3.1 (Nimagen), 0.15 µl of a primer (10 pmol µl⁻¹), 2.0 µl of the purified PCR product, and 5.85 µl of ddH₂O. Sequencing settings were: an initial denaturation at 96°C for 1 min, followed by 25 cycles of denaturation at 96°C for 10 sec, annealing at 55°C for 5 sec, and elongation at 60°C for 4 min (28S rRNA, COI and ITS-2) or 8 min (18S rRNA). In order to obtain good reads and avoid reading errors, sequencing reactions were carried out in both directions. Sequencing products were then purified with the ExTerminator kit (A&A Biotechnology) and suspended in 25 µl of formamide. Sequencing products were read with the ABI 3130xl sequencer at the Molecular Ecology Lab, Institute of Environmental Sciences of the Jagiellonian University, Kraków, Poland. Sequences were processed in BioEdit ver. 7.2.5 (Hall 1999).

Obtained sequences for the four DNA fragments were checked using Basic Local Alignment Search Tool (BLAST; Altschul *et al.* 1990) to verify eventual identity with sequences deposited in GenBank. In order to compare molecular similarity of the new species with other taxa, sequences from several other species belonging to the *Macrobiotus hufelandi* group were used (Table 2).

All sequences were aligned with the ClustalW Multiple Alignment tool (Thompson *et al.* 1994) implemented in BioEdit. Then, the aligned sequences were trimmed to: 328 (ITS-2), 621 (COI), 710 (28S rRNA), 808 (18S rRNA) bp. Uncorrected pairwise genetic distances were calculated using MEGA version 4 (Tamura *et al.* 2007).

Raw data underlying the description of *Macrobiotus polypiformis* sp. nov. are deposited in the Tardigrada Register (Michalczyk & Kaczmarek 2013) under www.tardigrada.net/register/0038.htm (and in the Supplementary Data). DNA sequences were deposited in GenBank (www.ncbi.nlm.nih.gov/genbank).

Table 2. Sequences used for molecular comparison of *Macrobiotus polypiformis* sp. nov. with several other species from the *Macrobiotus hufelandi* group.

DNA marker	Species	Accession number	Source
ITS-2	<i>Ma. polonicus</i> Pilato <i>et al.</i> , 2003	HM150647	Welnicz <i>et al.</i> 2011
	<i>Ma. sapiens</i> Binda & Pilato, 1984	GQ403680	Schill <i>et al.</i> 2010
	<i>Ma. paulinae</i> Stec <i>et al.</i> , 2015	KT935500	Stec <i>et al.</i> 2015
COI	<i>Ma. cf. hufelandi</i>	HQ876589-94, HQ876596	Guidetti <i>et al.</i> 2013
	<i>Ma. h. hufelandi</i> C.A.S. Schultze, 1834	HQ876584, HQ876586-8	Guidetti <i>et al.</i> 2013
	<i>Ma. macrocalix</i> Bertolani & Rebecchi, 1993	FJ176203-17, HQ876571	Guidetti <i>et al.</i> 2013
	<i>Ma. kristenseni</i> Guidetti <i>et al.</i> , 2013	KC193575-6	Guidetti <i>et al.</i> 2013
	<i>Ma. vladimiri</i> Bertolani <i>et al.</i> , 2011	HM136931-4, HQ876568	Guidetti <i>et al.</i> 2013
	<i>Ma. terminalis</i> Bertolani & Rebecchi, 1993	JN673960, AY598775	Guidetti <i>et al.</i> 2013
	<i>Ma. sandrae</i> Bertolani & Rebecchi, 1993	HQ876566-70, HQ876572-83	Guidetti <i>et al.</i> 2013
	<i>Ma. paulinae</i> Stec <i>et al.</i> , 2015	KT951668	Stec <i>et al.</i> 2015
28S	<i>Ma. hufelandi</i> gr.	FJ435751, FJ435754-5	Guil & Giribet 2012
	<i>Ma. paulinae</i> Stec <i>et al.</i> , 2015	KT935501	Stec <i>et al.</i> 2015
18S	<i>Ma. h. hufelandi</i> C.A.S. Schultze, 1834	GQ849024, X81442	Bertolani <i>et al.</i> 2014
	<i>Ma. hufelandi</i> gr.	HQ604971, FJ435738-40	Bertolani <i>et al.</i> 2014
	<i>Ma. kristenseni</i> Guidetti <i>et al.</i> , 2013	KC193577	Bertolani <i>et al.</i> 2014
	<i>Ma. macrocalix</i> Bertolani & Rebecchi, 1993	HQ604976	Bertolani <i>et al.</i> 2014
	<i>Ma. polonicus</i> Pilato <i>et al.</i> , 2003	HM187580	Bertolani <i>et al.</i> 2014
	<i>Ma. sapiens</i> Binda & Pilato, 1984	DQ839601	Bertolani <i>et al.</i> 2014
	<i>Ma. joannae</i> Pilato & Binda, 1983	HQ604974-5	Bertolani <i>et al.</i> 2014
	<i>Ma. paulinae</i> Stec <i>et al.</i> , 2015	KT935502	Stec <i>et al.</i> 2015

Abbreviations

LM = light microscopy

MECN = Museo Ecuatoriano de Ciencias Naturales, Sección de Entomología, Rumipamba 341 y Av. de los Shyris, Quito, Ecuador

DATE = Department of Animal Taxonomy and Ecology, Institute of Environmental Biology, Adam Mickiewicz University in Poznań, Umultowska 89, 61-614 Poznań, Poland

ZMUC = Zoological Museum, Natural History Museum of Denmark, University of Copenhagen, Universitetsparken 15, DK-2100 Copenhagen Ø, Denmark

Results

Phylum Tardigrada Doyère, 1840
Class Eutardigrada Richters, 1926
Order Parachela Schuster, Nelson, Grigarick & Christenberry, 1980
Superfamily Macrobiotioidea Thulin, 1928 (in Marley *et al.* 2011)
Family Macrobiotidae Thulin, 1928
Genus *Macrobiotus* C.A.S. Schultze, 1834

***Macrobiotus polypiformis* sp. nov.**

urn:lsid:zoobank.org:act:3858645A-2909-4DB9-82CD-CF2B4D7051C2

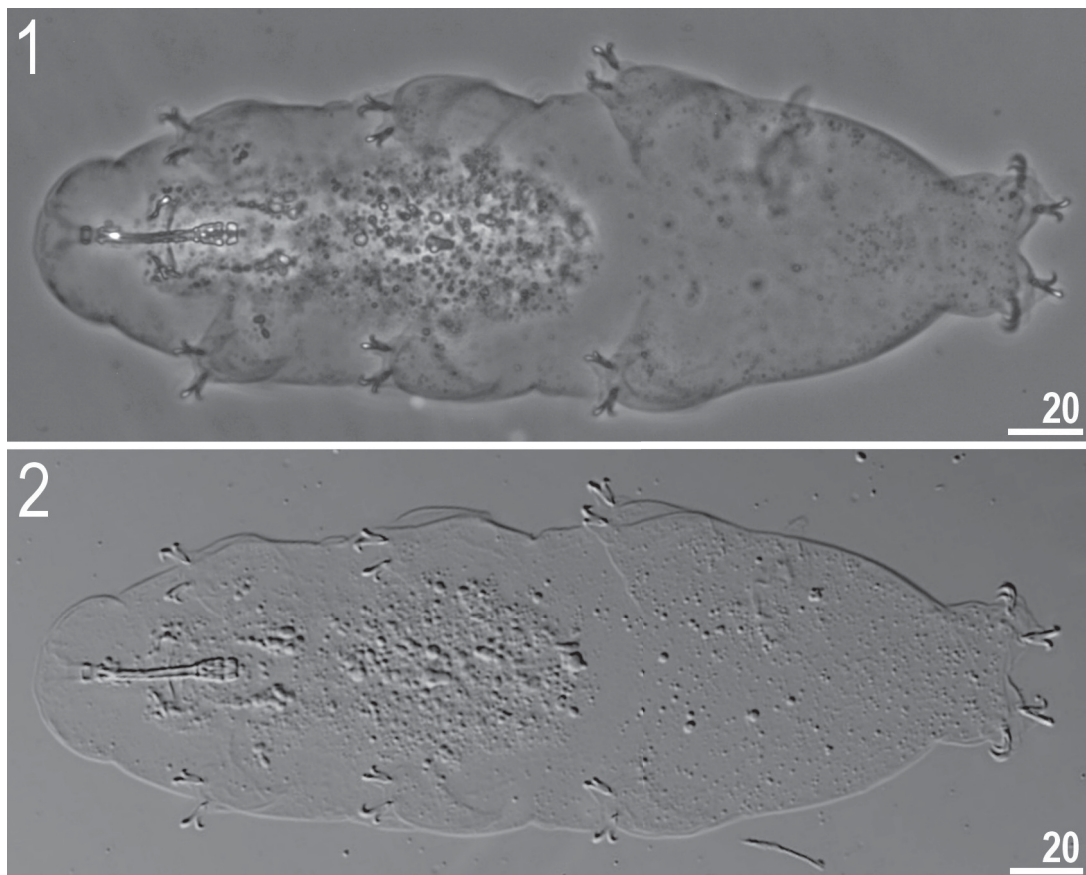
Figs 1–29; Tables 3–4

Etymology

The specific epithet ‘*polypiformis*’ refers to the similarity of the egg processes to the polyp form found in the phylum Cnidaria.

Material examined

Specimens mounted on microscope slides in Hoyer’s medium, fixed on SEM stubs or processed for DNA sequencing.



Figs 1–2. *Macrobiotus polypiformis* sp. nov., holotype, habitus, dorso-ventral projection. 1. Seen in PCM. 2. Seen in DIC. Scale bars in μm .

ROSZKOWSKA M. *et al.*, *Macrobiotus polypiformis* sp. nov. from Ecuador**Table 3.** Measurements (in μm) of selected morphological structures of individuals of *Macrobiotus polypiformis* sp. nov. mounted in Hoyer's medium (N = number of specimens/structures measured; Range = the smallest and the largest structure among all measured specimens; SD = standard deviation).

Character	N	Range		Mean		SD		Holotype	
		μm	pt	μm	pt	μm	pt	μm	pt
Body length	30	237–375	–	327		57		268	–
Buccopharyngeal tube									
Buccal tube length	30	24.4–32.5	–	29.2	–	2.5	–	26.4	–
Stylet support insertion point	30	17.1–23.5	70.1–72.9	20.9	71.6	1.9	0.9	18.9	71.6
Buccal tube external width	29	2.8–4.0	11.0–13.0	3.4	11.8	0.4	0.6	2.9	11.0
Buccal tube internal width	29	1.6–2.4	6.1–8.6	2.0	6.9	0.3	0.6	1.6	6.1
Ventral lamina length	26	13.5–17.3	52.1–55.1	15.8	53.4	1.3	1.0	14.0	53.0
Placoid lengths									
Macroplacoid 1	30	5.2–6.8	19.2–23.5	6.2	21.3	0.7	1.3	5.6	21.2
Macroplacoid 2	30	2.8–4.1	11.4–14.5	3.6	12.4	0.5	0.8	3.2	12.1
Microplacoid	29	1.5–2.3	5.8–7.1	1.8	6.3	0.2	0.4	1.6	6.1
Macroplacoid row	30	9.0–11.8	34.3–39.9	10.8	36.9	1.1	1.8	9.6	36.4
Placoid row	29	11.1–14.5	41.4–49.0	13.0	44.4	1.4	2.2	11.7	44.3
Claw 1 lengths									
External primary branch	28	8.2–11.5	31.4–38.1	10.2	34.8	1.0	1.5	9.7	36.7
External secondary branch	27	6.6–8.7	25.0–29.8	8.0	27.3	0.8	1.6	7.8	29.5
Internal primary branch	28	7.7–10.6	30.0–35.4	9.5	32.5	0.9	1.3	8.7	33.0
Internal secondary branch	25	6.0–8.6	24.3–28.3	7.9	26.6	0.8	1.3	6.9	26.1
Claw 2 lengths									
External primary branch	30	8.9–12.7	36.0–42.3	11.0	37.8	1.1	1.7	9.6	36.4
External secondary branch	30	6.8–9.4	27.0–32.6	8.5	29.1	0.8	1.8	7.7	29.2
Internal primary branch	30	7.8–11.0	30.6–35.1	9.6	32.9	0.9	1.3	8.6	32.6
Internal secondary branch	25	6.3–8.7	24.1–28.6	7.8	26.7	0.7	1.3	7.0	26.5
Claw 3 lengths									
External primary branch	30	8.9–12.7	36.1–41.9	11.1	38.0	1.0	1.3	9.9	37.5
External secondary branch	28	7.0–9.5	27.7–31.9	8.7	29.6	0.8	1.4	8.1	30.7
Internal primary branch	28	7.9–11.1	31.9–37.1	9.8	33.8	1.0	1.3	8.5	32.2
Internal secondary branch	24	6.5–9.7	26.2–31.6	8.4	28.5	0.9	1.7	7.0	26.5
Claw 4 lengths									
Anterior primary branch	29	8.6–12.7	35.2–42.6	11.2	38.4	1.3	2.1	10.1	38.3
Anterior secondary branch	26	7.1–10.5	27.1–33.4	9.0	30.4	1.0	2.1	7.2	27.3
Posterior primary branch	29	9.5–14.0	38.3–44.0	12.1	41.4	1.3	1.7	10.8	40.9
Posterior secondary branch	29	7.1–10.0	26.0–33.8	8.9	30.5	0.8	2.1	8.7	33.0

Holotype

ECUADOR: slide 1215/19, 3 Jan. 2015, Milena Roszkowska and Łukasz Kaczmarek leg. (MECN).

Paratypes

ECUADOR: 135 animals (including 10 simplex), one exuvia and 44 eggs (including two with developed embryos), same data as holotype (MECN, slide 1215/19 (with holotype), 4 paratypes (slides: 1215/*, where the asterisk can be substituted by any of the following numbers: 23, 25) and two eggs (slides: 1215/*: 8, 9); DATE, 92 paratypes (slides: 1215/*: 1, 2, 3, 10, 11, 12, 13, 14, 15, 16, 19, 20, 22, 24) and 24 eggs (slides: 1215/*: 1, 4, 5, 7, 26, 27); ZMUC, 5 paratypes (slides 1215/*: 17, 21) and 3 eggs (slide 1215/6)).

Type locality

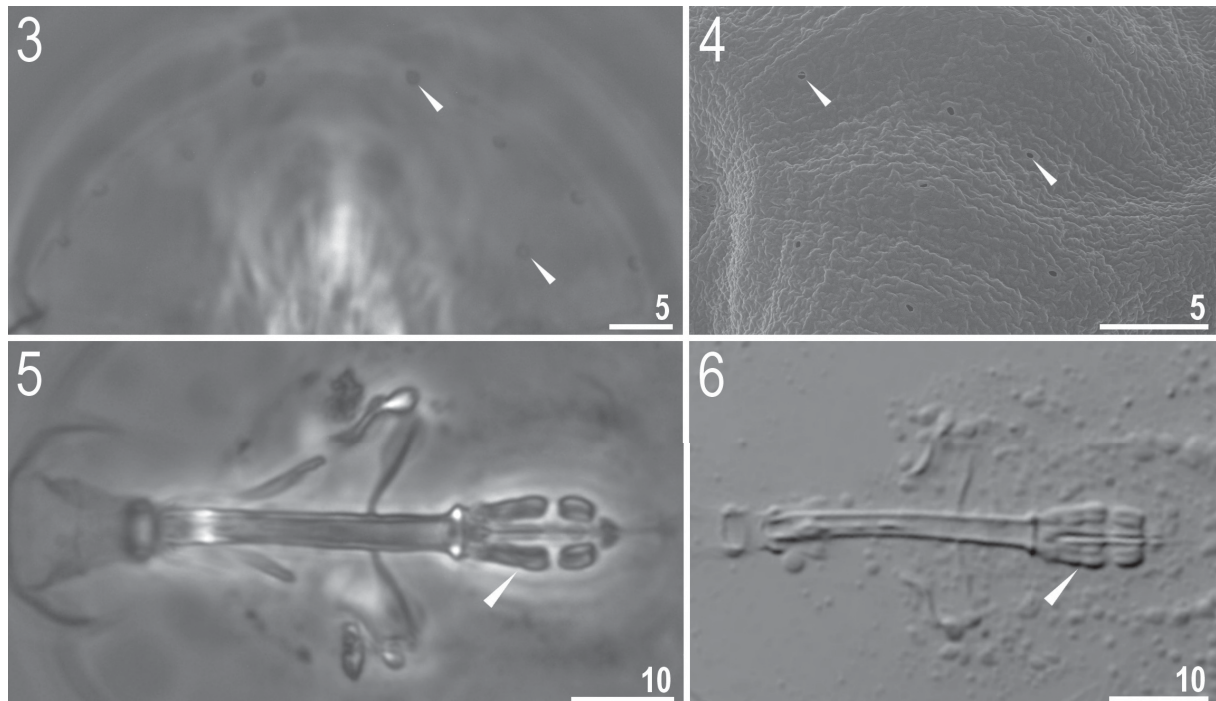
ECUADOR: Manabí Province, 1°04'06" S, 89°52'18" W; 370 m asl, moss sample from a concrete wall, next to E15 road, ca 3.5 km W from San Lorenzo, tropical rainforest.

Description

Animals (measurements and statistics in Table 3)

Body white in juveniles and adults, transparent after fixation in Hoyer's medium (Figs 1–2). Eyes present (in 93% of measured specimens). Dorsal and ventral cuticle smooth under LM. Additionally, small oval and round pores (0.9–1.2 µm in diameter), sometimes difficult to observe under LM, are scattered randomly on the entire cuticle (Figs 3–4). A ring of pores, difficult to observe under LM, is present around the mouth opening, below the peribuccal sensory lobes. One patch of fine and dense granulation above claws on legs I–IV present (Figs 16–19).

Mouth antero-ventral. Bucco-pharyngeal apparatus of the *Macrobotus* type (Figs 5–6), with the ventral lamina and ten small peribuccal lamellae (Figs 6, 9) followed by six buccal sensory lobes. Oral cavity armature composed of three bands of teeth, of which only the third band is visible under LM (Fig. 7, empty arrowhead). SEM is required to reveal the first and the second bands of teeth (see Figs 8–9). The first band of teeth comprises extremely small cones arranged in 3–4 rows situated at the anterior portion of the oral cavity, at the base of the peribuccal lamellae (Figs 8–9, filled arrowheads). The second band of teeth is composed of 4–5 rows of small cones (but larger than those on the first band), positioned towards the rear of the oral cavity, between the ring fold and the third band of teeth (Figs 8–9, arrows). The teeth of the third band are positioned at the rear of the oral cavity, between the second band of teeth and the buccal tube opening (Figs 7–9, empty arrowheads).



Figs 3–6. *Macrobotus polypiformis* sp. nov. **3.** Cuticular pores visible in PCM (paratype). **4.** Cuticular pores visible in SEM (paratype). **5–6.** Bucco-pharyngeal apparatus (dorso-ventral projection), the filled arrowhead indicates the first macroplacoid with central constriction (Fig. 5 = paratype in PCM; Fig. 6 = holotype in DIC). Scale bars in µm.

Under LM, the teeth of the third band appear as a single, thin, transversal ridge both ventrally and dorsally (Fig. 7, empty arrowhead). Although SEM reveals that both ventral and dorsal teeth do indeed form continuous ridges, there are evident median (M) and lateral (L) peaks corresponding to the median and lateral teeth found in species with better developed oral cavity armatures (Figs 8–9). Median teeth are smaller than the lateral teeth (Fig. 8). In addition, there are a number of smaller accessory teeth (a) placed laterally to the lateral teeth. These accessory teeth are better developed ventrally than dorsally (Fig. 8). Pharyngeal bulb spherical, with triangular apophyses, two rod-shaped macroplacoids and a triangular microplacoid (Figs 5–6). The macroplacoid length sequence $1 > 2$. The first macroplacoid with a central constriction (Figs 5–6, the filled arrowhead).

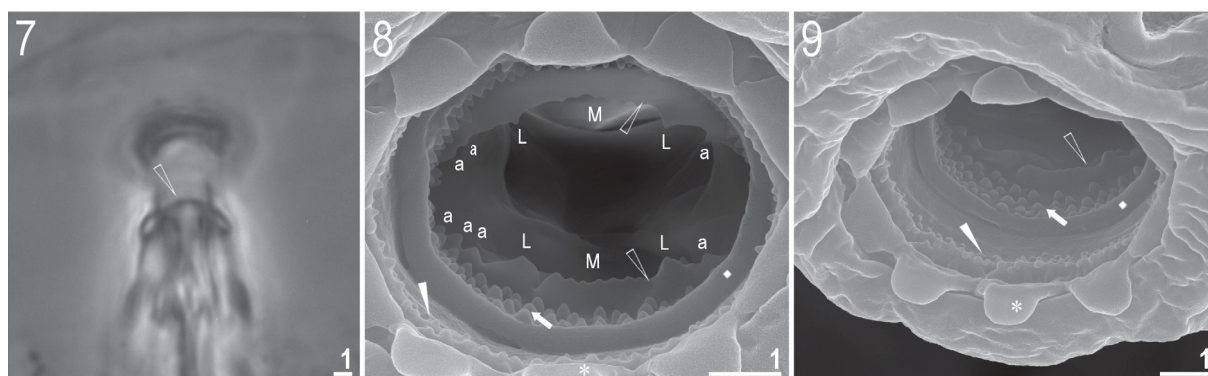
Claws small and slender of the *hufelandi* type (Figs 10–14). Primary branches with distinct accessory points. Lunules on legs I–III smooth (Figs 10, 13), those on legs IV dentate (Figs 12, 14). Bars under claws absent but paired muscle attachments below claws I–III present (hardly visible under LM and only slightly more visible under SEM).

Eggs (measurements and statistics in Table 4)

Laid freely, white to light yellow, spherical and with a *hufelandi* type chorion ornamentation (Figs 20–28). The surface between processes is covered with a dense regular reticulum (mesh diameter 0.5–0.8 μm) (Figs 22, 25–28). Processes in the shape of inverted goblets with slightly concave, conical, micro-granulated trunks and well-defined terminal discs (Figs 22–23, 25–26, 28). When observed under SEM, some process trunks have 3–5 faint, annular ring undulations (Figs 26, 28), whereas in LM these undulations are not visible. Terminal discs are cog-shaped, with each of the 8–10 ‘cog-teeth’ extended to form a long, thin, hair-like and flexible filament that probably serves to enhance the adhesive function of egg processes. Under SEM, small rounded granules or aggregations of granules, 0.06–0.15 μm in diameter, are visible on the filaments (Fig. 29). Central area of the terminal disc with sparse, randomly distributed, small granules (Figs 25, 28). Moreover, the area between the granules on the filaments, the surface of the terminal disk and the trunk of the processes appear, under SEM, to be micro-granulated (Figs 28–29).

DNA sequences

Initially, four molecular markers obtained from four of the eight individuals were sequenced. The sequences from 18S rRNA, 28S rRNA, ITS-2 exhibited a lack of polymorphism, whereas for COI two distinct haplotypes were obtained. Knowing that ITS-2 and COI have relatively high mutation rates,



Figs 7–9. *Macrobiotus polypiformis* sp. nov., paratypes. 7. Third band of teeth visible under PCM (empty arrowhead). 8–9. Oral cavity armature (SEM), the filled arrowheads indicate teeth of the first band, arrows indicate teeth of the second band, the empty arrowheads indicate teeth of the third band, M = median teeth, L = lateral teeth, a = accessory teeth, rhombi = ring folds, asterisks = ventral side.

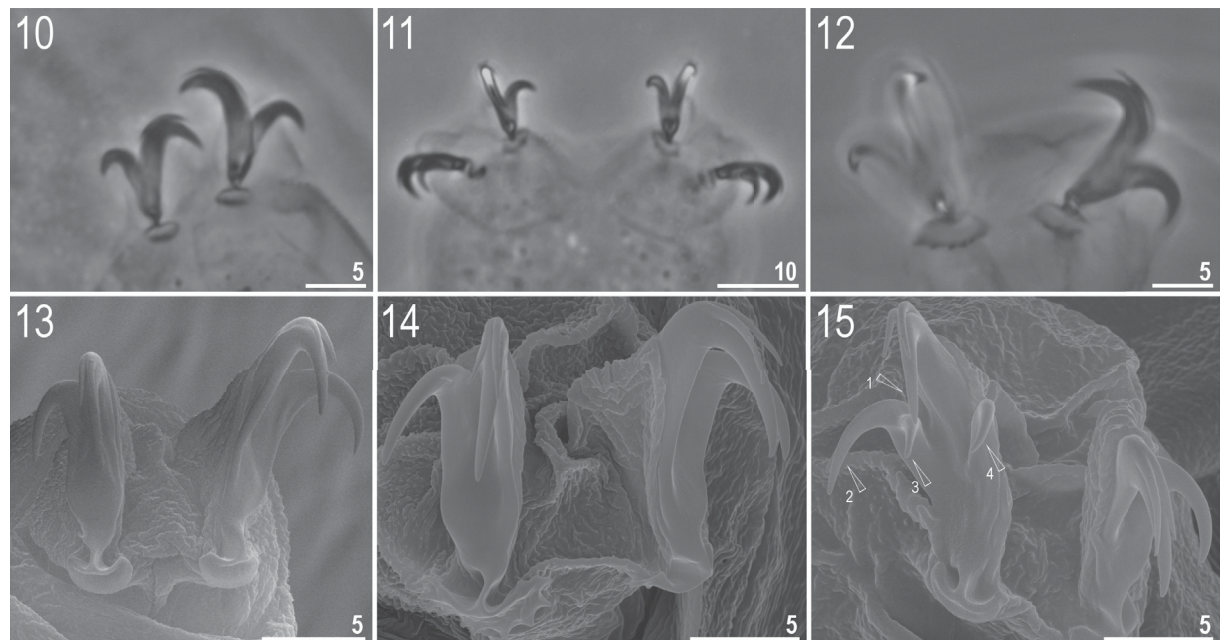
Table 4. Measurements (in μm) of selected morphological structures of eggs of *Macrobiotus polypiformis* sp. nov. mounted in Hoyer's medium (N = number of eggs/structures measured; Range = the smallest and the largest structure among all measured specimens; SD = standard deviation).

Character	N	Range	Mean	SD
Diameter of egg without processes	20	61.9–70.5	66.7	2.2
Diameter of egg with processes	16	70.4–81.2	76.7	2.8
Process height	60	4.3–6.5	5.5	0.5
Process base width	60	3.3–5.9	4.5	0.5
Process base/height ratio	42	69%–91%	80%	6%
Terminal disc width	60	2.7–5.1	3.9	0.5
Distance between processes	60	3.0–7.0	4.8	0.9
Number of processes on the egg circumference	18	19–23	21.1	1.5

these fragments were sequenced for the remaining four specimens. The eight individuals were found to have identical ITS-2 sequences while COI revealed two haplotypes (frequency 1:1) differing by 20 substitutions. One consensus sequence for each nuclear marker and one consensus sequence for each COI haplotype were deposited in GenBank: 18S rRNA sequence, 1726 bp long (GenBank accession number: KX810008), 28S rRNA sequence, 725 bp long (KX810009), COI sequence for *haplotype 1*, 658 bp long (KX810011), COI *haplotype 2*, 658 bp long (KX810012), and ITS-2 sequence, 425 bp long (KX810010).

Remark

Among the 136 individuals of the new species, one specimen had abnormal claws, i.e., internal and external claw II and III on one leg had additional secondary branches (Figs 15, 19). Additionally, on the



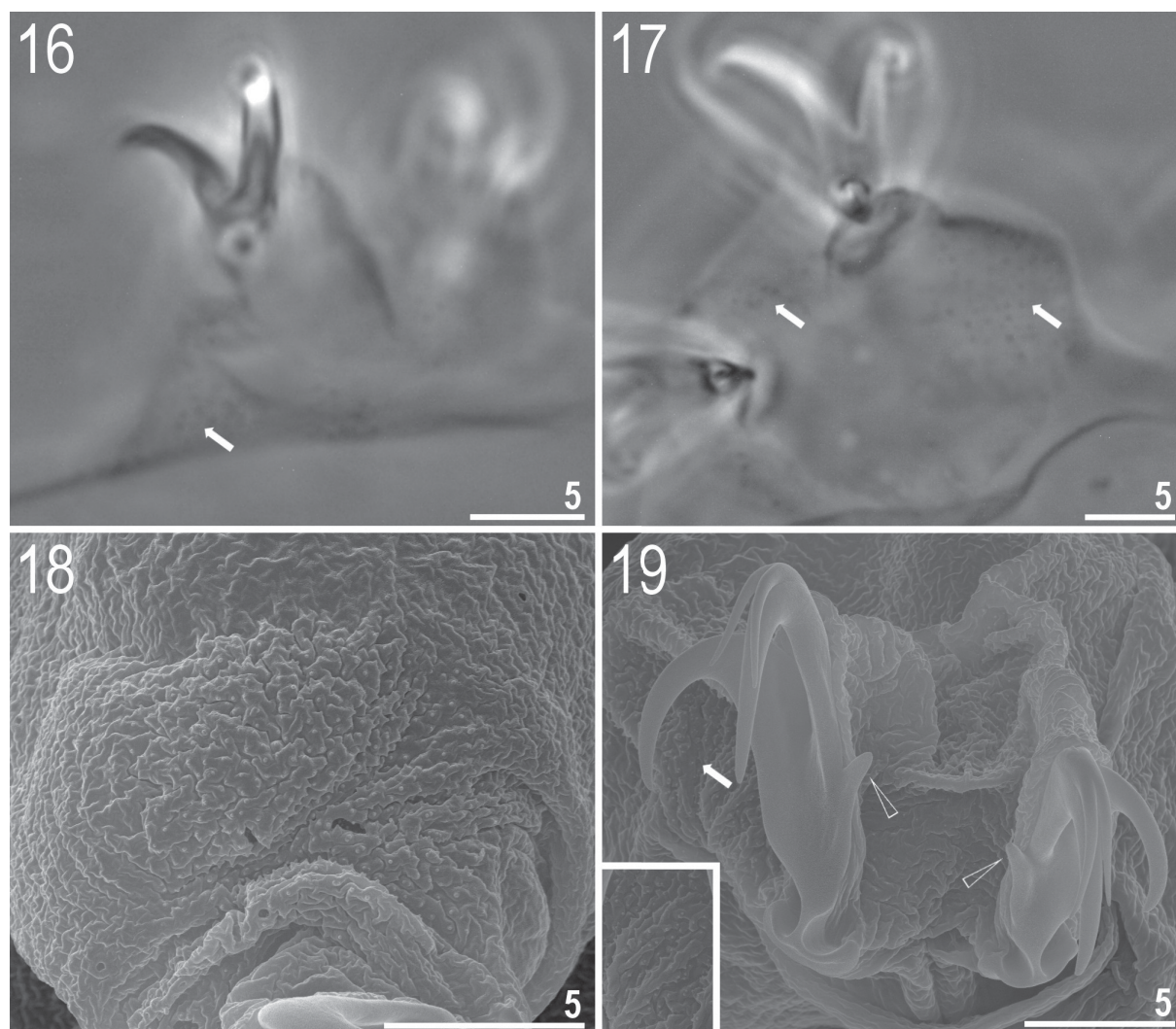
Figs 10–15. Claws. **10.** Claw II with smooth lunules (PCM, paratype). **11.** Claw IV (PCM, holotype). **12.** Indented lunules on claw IV (PCM, paratype). **13.** Claw I with smooth lunules (SEM, paratype). **14.** Claw IV with indented lunules (SEM, paratype). **15.** Aberrant claw IV (SEM, paratype). Numbers 1–2 indicate normally developed claw branches/spurs, whereas 3–4 indicate aberrant claw branches/spurs. Scale bars in μm .

claws of the IV pair of legs, unique, upwardly turned spurs were present near the lower half of the claw (Fig. 19).

Differential diagnosis

Morphological

Macrobiotus polypiformis sp. nov., by the presence of long, flexible filaments on the egg processes and faint annular undulations on their trunks, is most similar to *Ma. paulinae* Stec *et al.*, 2015, but differs from it by possessing larger pores on the cuticle ($0.9\text{--}1.2\text{ }\mu\text{m}$ in *Ma. polypiformis* sp. nov. vs $0.3\text{--}0.5\text{ }\mu\text{m}$ in *Ma. paulinae*), smooth cuticle (seven dorso-lateral patches of sparse granulation arranged symmetrically on both sides of the body in *Ma. paulinae*), one patch of granulation on legs I–IV (two distinct patches of granulation, fine and dense granulation above claws and a more robust and sparse granulation in the middle of each leg in *Ma. paulinae*), a different number of rows of teeth in the first band of teeth in the oral cavity (3–4 rows in *Ma. polypiformis* sp. nov. vs a single row in *Ma. paulinae*), larger reticulum mesh diameter covering the egg surface ($0.5\text{--}0.8\text{ }\mu\text{m}$ in *Ma. polypiformis* sp. nov. vs

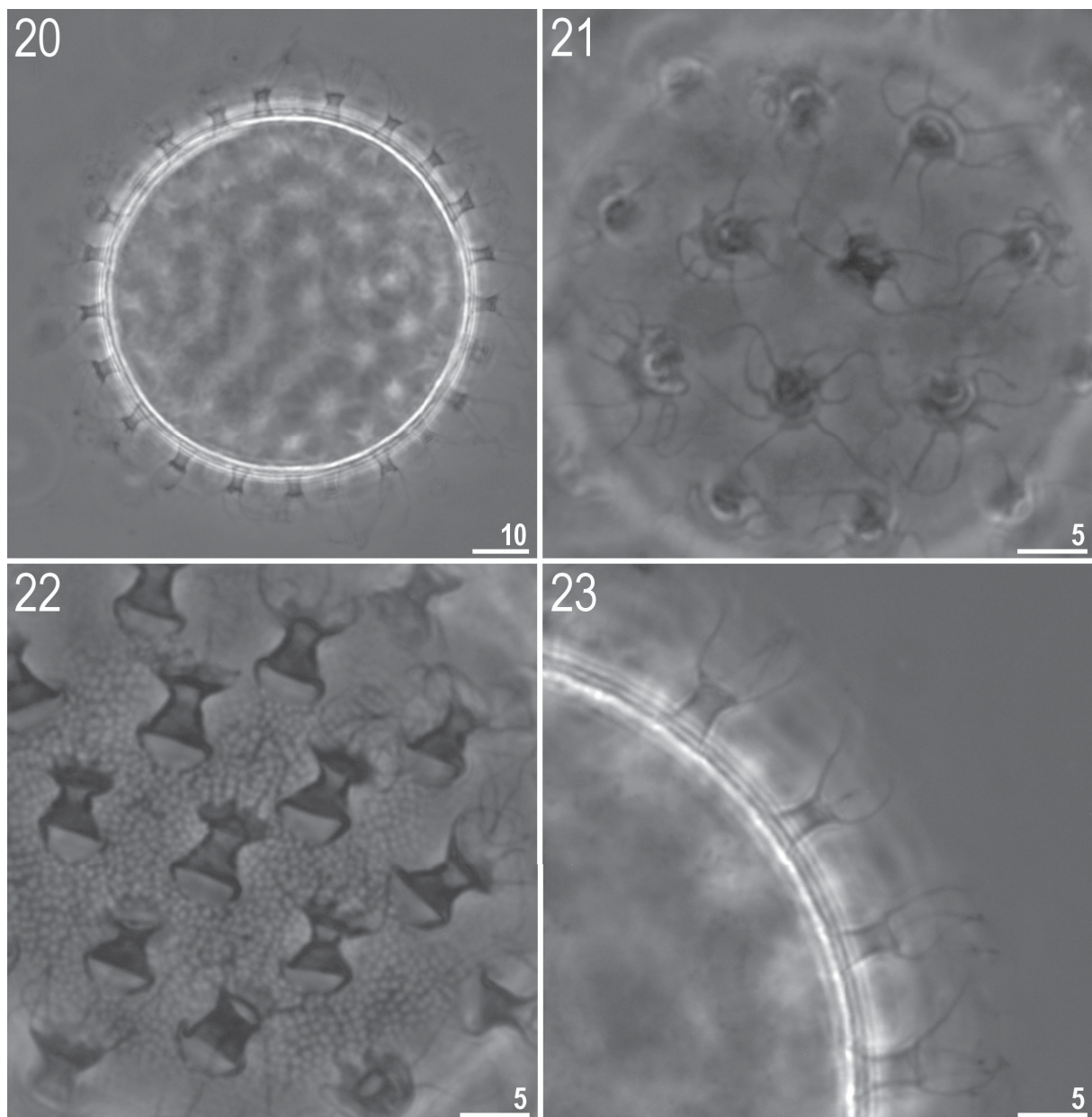


Figs 16–19. *Macrobiotus polypiformis* sp. nov., paratypes. **16.** Granulation on the II pair of legs, arrow (PCM). **17.** Granulation on IV pair of legs, arrow (PCM). **18.** Granulation on II pair of legs (SEM). **19.** Modified claw IV (arrowheads) and granulation on IV pair of legs, arrow (SEM). Scale bars in μm .

0.05–0.2 μm in *Ma. paulinae*), trunks of the egg processes with faint annular undulations (distinct and clearly visible in *Ma. paulinae*), different morphology of the terminal disc margins (8–10 long, hair-like, flexible filaments in *Ma. polypiformis* sp. nov. vs small, irregular teeth, instead of filaments, in *Ma. paulinae*, with only some processes having one to a few flexible filaments), lower number of processes on the egg circumference (19–23 in *Ma. polypiformis* sp. nov. vs 24–32 in *Ma. paulinae*).

Genotypic

It was confirmed, using Basic Local Alignment Search Tool (BLAST; Altschul *et al.* 1990), that no sequences deposited in GenBank were identical with sequences obtained from the type population of



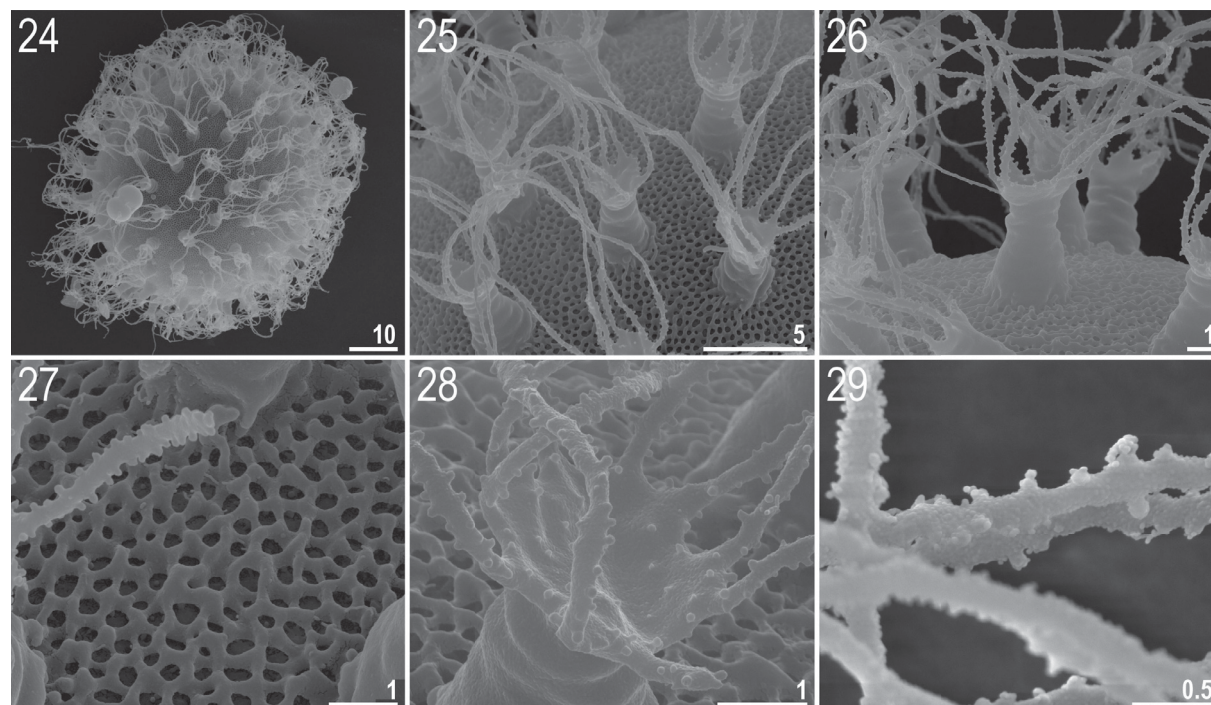
Figs 20–23. *Macrobiotus polypiformis* sp. nov. **20.** Chorion. **21.** Long, hair-like flexible filaments on terminal discs. **22.** Egg processes and the surface between egg processes with reticular design. **23.** Egg processes with filaments. All in PCM. Scale bars in μm .

Ma. polypiformis sp. nov. The p-distances calculated for ITS-2 between the new species and *Ma. polonicus* Pilato, Kaczmarek, Michalczyk & Lisi, 2003, *Ma. spaiens* Binda & Pilato, 1984 and *Ma. paulinae* are 39.4%, 25.6% and 31.1%, respectively. In the case of COI, genetic distances between the two haplotypes of *Ma. polypiformis* sp. nov. is 3.1%, which is above the threshold for species delineation proposed by Cesari *et al.* (2009). However, sequences from haplotype 1 and 2 are clearly distant from sequences of each species used in the analysis. For haplotype 1, p-distance range from 20.6% to 25.8%; haplotype 2 from 21.0% to 25.9%. In each case the most similar to the new species is *Ma. paulinae* (KT951668) and the most distinct is *Ma. macrocalix* Bertolani & Rebecchi, 1993 (HQ876571, FJ176208-12). Given there is no polymorphism in the ITS-2 sequences coming from the type population and the morphologically most similar species *Ma. paulinae* differs from the new species by a genetic distance of nearly 21%, it can be claimed that *Ma. polypiformis* sp. nov. is a valid new species exhibiting two COI haplotypes. P-distances for more conservative DNA fragments (28S rRNA and 18S rRNA) are, as was expected, lower than for more variable markers (COI and ITS-2). The range for 28S rRNA is 7.3%–12.9% with the most similar species *Ma. paulinae* (KT935501) and for 18S rRNA 3.0%–6.2% where the closest related species are *Ma. paulinae* (KT935502) and *Ma. sapiens* (DQ839601).

Discussion

Remarks on claw abnormalities and taxonomic status of *Me. armatus* Pilato & Binda, 1996

The morphological abnormalities of some body parts (e.g., shape and size of shells, number, length and shape of body appendages or claws) in many groups of invertebrates, are reported quite frequently. However, the percentage of abnormally developed specimens is rather low in normal environmental conditions, but can be much higher in polluted environments (e.g., Geslin *et al.* 2002). The stress factors proposed as possible sources of higher rates of appearance of morphological abnormalities are



Figs 24–29. *Macrobiotus polypiformis* sp. nov. **24.** Chorion. **25.** Long, hair-like flexible filaments on terminal discs. **26.** Egg processes with faint annular undulations. **27.** Surface between egg processes with reticular design. **28.** Terminal discs with small, randomly arranged granules. **29.** Small granules on filaments. All in SEM. Scale bars in μm .

different types of pollution, radioactivity, and changes in salinity or heavy metals. Additionally, such morphological abnormalities were also proposed as a possible source of taxonomical problems and misidentifications (e.g., De Oliveira Dias 1999; Reinert 1999; Samir & El-Din 2001; Elmoor-Loureiro 2004; Meriç *et al.* 2008; Zagal 2008; Głowska & Skoracki 2009; Fransozo *et al.* 2012; Miličić *et al.* 2013; Méndez-de Daboín *et al.* 2015).

The number of claws and their morphology are very important in tardigrade taxonomy at the family, genus, and species levels. The presence or absence of accessory points, lunules (dentate or smooth) or spurs, as well as the length of all structures (e.g., of primary and secondary branches and spurs) together with their shapes, are very important at the species level in most tardigrade genera (e.g., Pilato & Binda 2010; Marley *et al.* 2011). In most cases these structures are very conservative on the species level. However, some claw modifications, or rather abnormalities, have been observed in e.g., *Ma. naskreckii* Bąkowski *et al.*, 2016, *Milnesium* sp. (reported as *Mi. tardigradum* Doyère, 1840), *Mi. beasleyi* Kaczmarek, Jakubowska & Michalczyk, 2012 or *Mi. reductum* Tumanov, 2006 (Dastyh 1984; Tumanov 2006; Michalczyk *et al.* 2012; Bąkowski *et al.* 2016). Similar abnormalities can be observed in *Ma. polypiformis* sp. nov. (Figs 15, 19). Sometimes such abnormalities are easy to recognise as in *Ma. polypiformis* sp. nov. (Figs 15, 19), *Milnesium* sp. (Dastyh 1984: fig. 33), *Mi. reductum* (Tumanov 2006: figs 35–36) or *Mi. beasleyi* (Kaczmarek *et al.* 2012: fig. 11) as these additional spurs are usually much smaller, have a different shape, or are in an unusual place in comparison to other spurs on the claws of the same specimen. In other cases they can look like normally developed structures which are normal parts of the claws, especially when we observe only a single specimen instead of an entire population, as demonstrated in *Ma. naskreckii* (Bąkowski *et al.* 2016: figs 36–39) and *Ma. polypiformis* sp. nov. (Figs 15, 19). Unfortunately, specific studies focused on the presence of different kinds of abnormalities and their frequencies in tardigrades have never been conducted.

Mesobiotus armatus was described by Pilato & Binda (1996) from New Zealand (Gillespies Beach, South Island). The authors examined about 60 specimens and eggs from various localities in New Zealand, attributed earlier to *Me. liviae* (Ramazzotti, 1962) by Horning *et al.* (1978). These specimens were consequently attributed to *Me. diffusus* (Binda & Pilato, 1987), *Me. hieronimi* (Pilato & Claxton, 1988), *Me. montanus* (Murray, 1910) and a new species *Me. pseudoliviae* (Pilato & Binda, 1996). However, they found one very unusual specimen from Gillespies Beach, South Island, from which they described another new species, *Me. armatus*, mainly based on the presence of “spurs turned toward the apex” on the first three pairs of legs (Pilato & Binda 1996: fig. 4c). According to the authors, another character which differentiated *Me. armatus* from other *Mesobiotus* species was the lower *pt* value of the stylet support insertion point (*pt* 72.90). However, recent studies have shown that this *pt* value can be even lower in some species of this group (e.g., the minimum *pt* of the stylet support insertion point in *Me. pseudoblocki* Roszkowska *et al.*, 2016 is 71.5). The remaining characters in the description of *Me. armatus* were typical of other species within the genus *Mesobiotus*. Describing new species from genera like *Macrobotus*, *Mesobiotus* or *Paramacrobotus* without the eggs, which are critical to the correct identification of most of the species in these genera (e.g., Bertolani & Rebecchi 1993; Kaczmarek *et al.* 2011), is highly risky. However, in the case of *Me. armatus* the presence of a unique character, in the form of spurs on claws, was considered sufficient to describe the new species, even based on only a single specimen. Based on recently published data (Tumanov 2006; Kaczmarek *et al.* 2012; Bąkowski *et al.* 2016) and on the observations from the present paper (see Remarks to the new species and Figs 15, 19), we can hypothesize that the decision to describe *Me. armatus* was premature. As is shown in Fig. 19, the same type of “spurs” as present in *Me. armatus* were found in one specimen of *Ma. polypiformis* sp. nov. and similar abnormalities were also present in a single specimen of *Ma. naskreckii*. These findings suggest that the “spurs” found in *Me. armatus* may be an abnormality rather than a unique and constant character. This conclusion is further supported by the finding of only a single specimen of *Me. armatus* among the many other specimens of *Mesobiotus* (from many New Zealand

localities) with normally developed claws. We, therefore, suggest that *Me. armatus* be considered *species inquirenda* (i.e., of uncertain taxonomic status) until such time as its validity as a species can be confirmed. The case of *Me. armatus* exposes a wider problem with the practice of describing tardigrade species based on a limited number of specimens (in extreme cases, species have been erected on the basis of a single specimen). We can conclude that such descriptions should only be accepted when supported by appropriate nucleotide sequences. Without these sequences, such descriptions may serve only to exaggerate tardigrade diversity. Similar conclusions were reached by Stec *et al.* (2016b), who demonstrated considerable egg variability in *Ramazzottius subanomalous* (Biserov, 1985). Ultimately, we suggest that tardigradologists should avoid describing new species based on scarce material and especially on a single specimen. Moreover, we think that all species previously described on the basis of a single specimen, or without eggs (for those species belonging to the genera in which eggs are indispensable to the correct species identification), should be confirmed and redescribed based on a larger sample.

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