European Journal of Taxonomy 327: 1–19
https://doi.org/10.5852/ejt.2017.327

ISSN 2118-9773
www.europeanjournaloftaxonomy.eu
2017 · Roszkowska M. *et al.*



**Research article**

urn:lsid:zoobank.org:pub:C8F064E6-137C-4D50-A738-CCC828281CD1

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

Milena ROSZKOWSKA[1,*], Marta OSTROWSKA[2], Daniel STEC[3], Karel JANKO[4] & Łukasz KACZMAREK[5]

[1,5]Department of Animal Taxonomy and Ecology, Faculty of Biology, Adam Mickiewicz University in Poznań, Umultowska 89, 61-614 Poznań, Poland.
[2]Department of Avian Biology and Ecology, Faculty of Biology, Adam Mickiewicz University in Poznań, Umultowska 89, 61-614 Poznań, Poland.
[3]Department of Entomology, Institute of Zoology and Biomedical Research Jagiellonian University, Gronostajowa 9, 30-387 Kraków, Poland.
[4]Laboratory of Fish Genetics, Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, Rumburská 89, 277 21, Liběchov, Centre for Polar Ecology, Faculty of Science, University of South Bohemia in České Budějovice, Czech Republic.

[*]Corresponding author: mil.roszkowska@gmail.com
[2]Email: marta.ostrowska@amu.edu.pl
[3]Email: daniel_stec@interia.eu
[4]Email: janko@iapg.cas.cz
[5]Email: kaczmar@amu.edu.pl

[1]urn:lsid:zoobank.org:author:3C2055BD-866C-4B74-8669-A0749C6C9D7D
[2]urn:lsid:zoobank.org:author:727B2A95-C68B-4B5F-B732-8429A686AA47
[3]urn:lsid:zoobank.org:author:C148C15D-9F12-4312-9702-B1E1513C63CC
[4]urn:lsid:zoobank.org:author:DA3713CE-1E79-4DC4-9BBE-19CF7A0443E3
[5]urn:lsid:zoobank.org:author:7C4605F4-97C2-4EB8-93A2-C20598AFB63D

**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.

Roszkowska M., Ostrowska M., Stec D., Janko K. & Kaczmarek Ł. 2017. *Macrobiotus polypiformis* sp. nov., a new tardigrade (Macrobiotidae; *hufelandi* group) from the Ecuadorian Pacific coast, with remarks on the claw abnormalities in eutardigrades. *European Journal of Taxonomy* 327: 1–19. https://doi.org/10.5852/ejt.2017.327

## 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 (Dastych 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 *et al.* (2008) |
| | SSU82_R | reverse | TGATCCTTCTGCAGGTTCACCTAC | Sands *et al.* (2008) |
| 28S rRNA | 28SF0001 | forward | ACCCVCYNAATTTAAGCATAT | Mironov *et al.* (2012) |
| | 28SR0990 | reverse | CCTTGGTCCGTGTTTCAAGAC | Mironov *et al.* (2012) |
| COI | LCO1490 | forward | GGTCAACAAATCATAAAGATATTGG | Folmer *et al.* (1994) |
| | HCO2198 | reverse | TAAACTTCAGGGTGACCAAAAAATCA | Folmer *et al.* (1994) |
| ITS-2 | ITS3 | forward | GCATCGATGAAGAACGCAGC | White *et al.* (1990) |
| | ITS4 | reverse | TCCTCCGCTTATTGATATGC | White *et al.* (1990) |

specific genetic variation (e.g., Hebert *et al.* 2003; Cesari *et al*. 2009; Bertolani *et al.* 2011; Wełnicz *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 $Mg^{2+}$ 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 $\mu l^{-1}$), 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 | *Ma. polonicus* Pilato *et al*, 2003 | HM150647 | Wełnicz *et al.* 2011 |
| | *Ma. sapiens* Binda & Pilato, 1984 | GQ403680 | Schill *et al.* 2010 |
| | *Ma. paulinae* Stec *et al.*, 2015 | KT935500 | Stec *et al.* 2015 |
| COI | *Ma.*cf. *hufelandi* | HQ876589-94, HQ876596 | Guidetti *et al.* 2013 |
| | *Ma. h. hufelandi* C.A.S. Schultze, 1834 | HQ876584, HQ876586-8 | Guidetti *et al.* 2013 |
| | *Ma. macrocalix* Bertolani & Rebecchi, 1993 | FJ176203-17, HQ876571 | Guidetti *et al.* 2013 |
| | *Ma. kristenseni* Guidetti *et al.*, 2013 | KC193575-6 | Guidetti *et al.* 2013 |
| | *Ma. vladimiri* Bertolani *et al.*, 2011 | HM136931-4, HQ876568 | Guidetti *et al.* 2013 |
| | *Ma. terminalis* Bertolani & Rebecchi, 1993 | JN673960, AY598775 | Guidetti *et al.* 2013 |
| | *Ma. sandrae* Bertolani & Rebecchi, 1993 | HQ876566-70, HQ876572-83 | Guidetti *et al.* 2013 |
| | *Ma. paulinae* Stec *et al.*, 2015 | KT951668 | Stec *et al.* 2015 |
| 28S | *Ma. hufelandi* gr. | FJ435751, FJ435754-5 | Guil & Giribet 2012 |
| | *Ma. paulinae* Stec *et al.*, 2015 | KT935501 | Stec *et al.* 2015 |
| 18S | *Ma. h. hufelandi* C.A.S. Schultze, 1834 | GQ849024, X81442 | Bertolani *et al.* 2014 |
| | *Ma. hufelandi* gr. | HQ604971, FJ435738-40 | Bertolani *et al.* 2014 |
| | *Ma. kristenseni* Guidetti *et al.*, 2013 | KC193577 | Bertolani *et al.* 2014 |
| | *Ma. macrocalix* Bertolani & Rebecchi, 1993 | HQ604976 | Bertolani *et al.* 2014 |
| | *Ma. polonicus* Pilato *et al.*, 2003 | HM187580 | Bertolani *et al.* 2014 |
| | *Ma. sapiens* Binda & Pilato, 1984 | DQ839601 | Bertolani *et al.* 2014 |
| | *Ma. joannae* Pilato & Binda, 1983 | HQ604974-5 | Bertolani *et al.* 2014 |
| | *Ma. paulinae* Stec *et al.*, 2015 | KT935502 | Stec *et al.* 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 Macrobiotoidea Thulin, 1928 (in Marley *et al.* 2011)
Family Macrobiotidae Thulin, 1928
Genus *Macrobiotus* C.A.S. Schultze, 1834

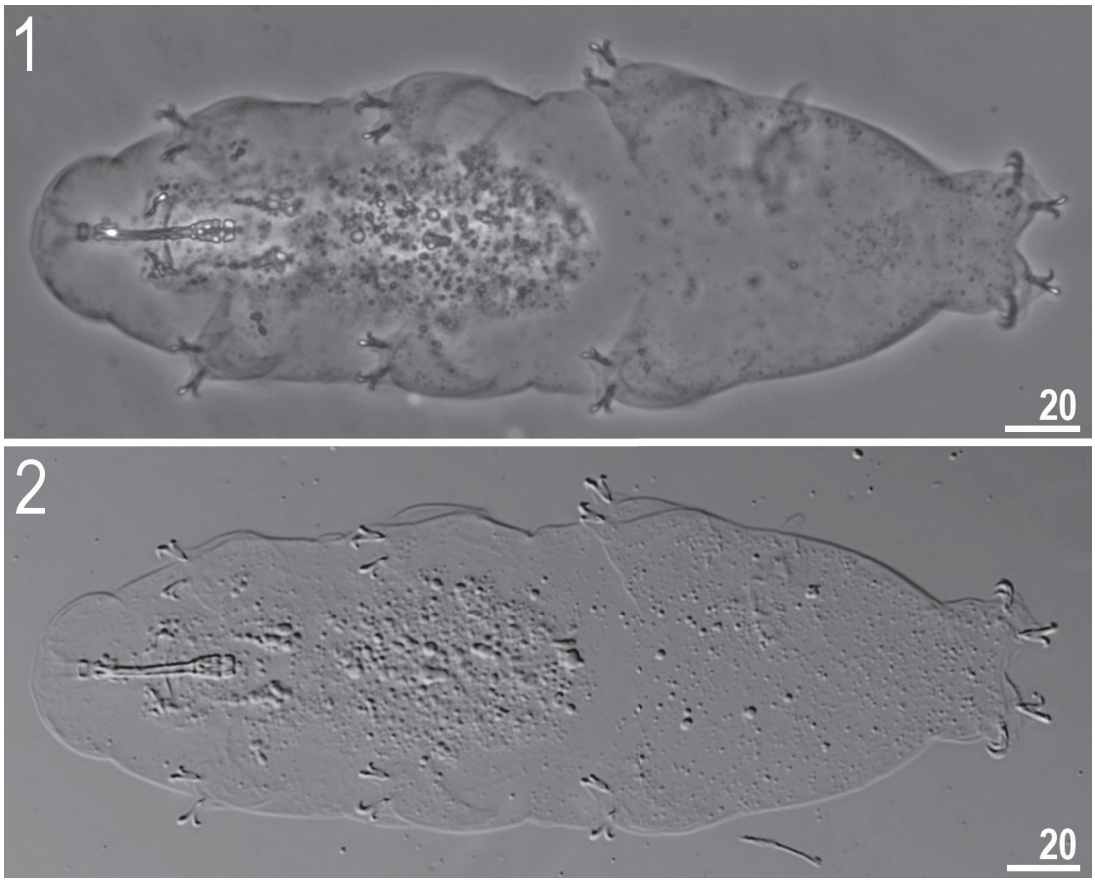
***Macrobiotus polypiformis*** sp. nov.
urn:lsid:zoobank.org:act:3858645A-2909-4DB9-82CD-CF2B4D7051C2
Figs 1–29; Tables 3–4

### 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.

**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**

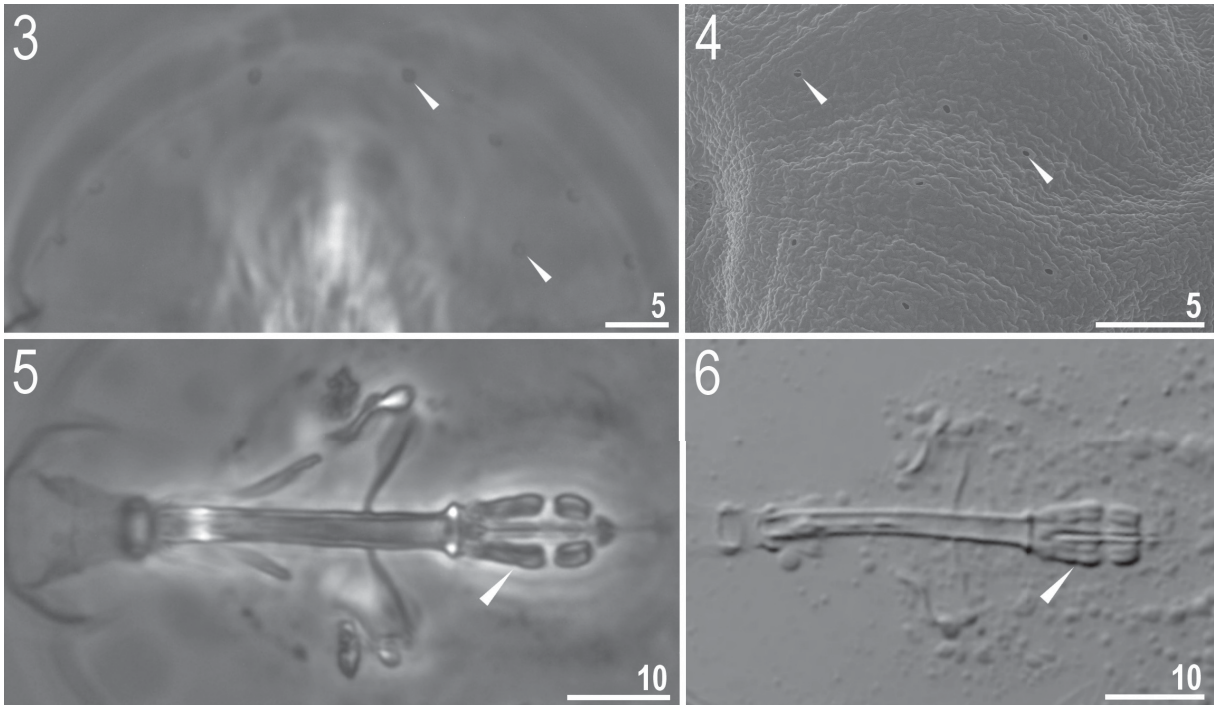
ECUADOR: Manabí Province, 1°04′06″ S, 89°52′18″ W; 370 m asl, moss sample from a concrete wall, next to E15 road, ca 3.5 km W from San Lorenzo, tropical rainforest.

**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 *Macrobiotus* 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.** *Macrobiotus 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).

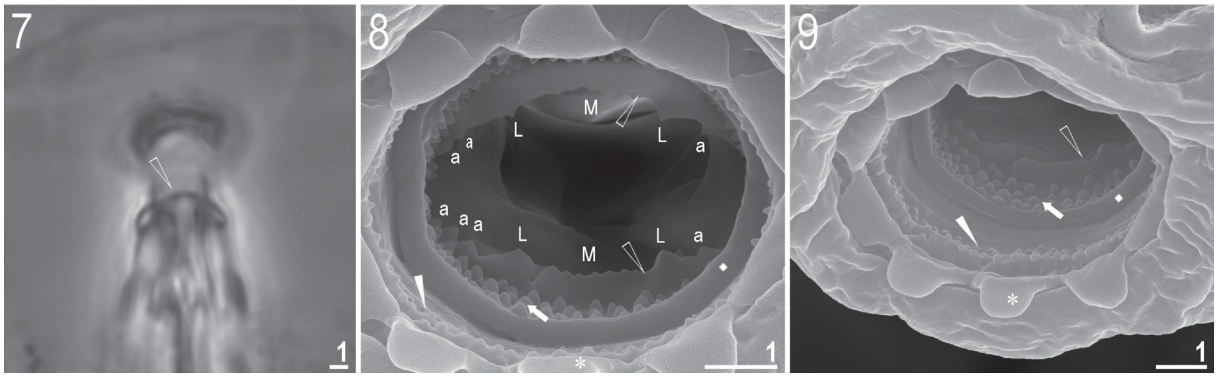
Claws small and slender of the *hufelandi* type (Figs 10–14). Primary branches with distinct accessory points. Lunules on legs I–III smooth (Figs 10, 13), those on legs IV dentate (Figs 12, 14). Bars under claws absent but paired muscle attachments below claws I–III present (hardly visible under LM and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

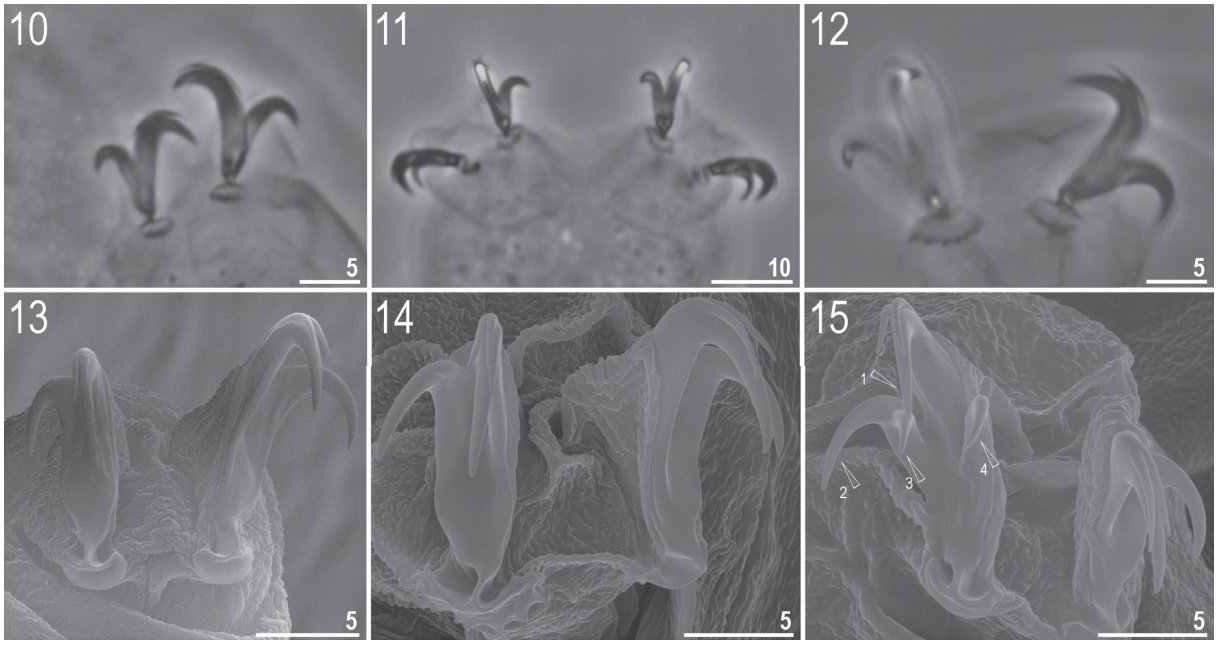
**Table 4.** Measurements (in µm) of selected morphological structures of eggs of *Macrobiotus polypiformis* sp. nov. mounted in Hoyer's medium (N = number of eggs/structures measured; Range = the smallest and the largest structure among all measured specimens; SD = standard deviation).

| 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



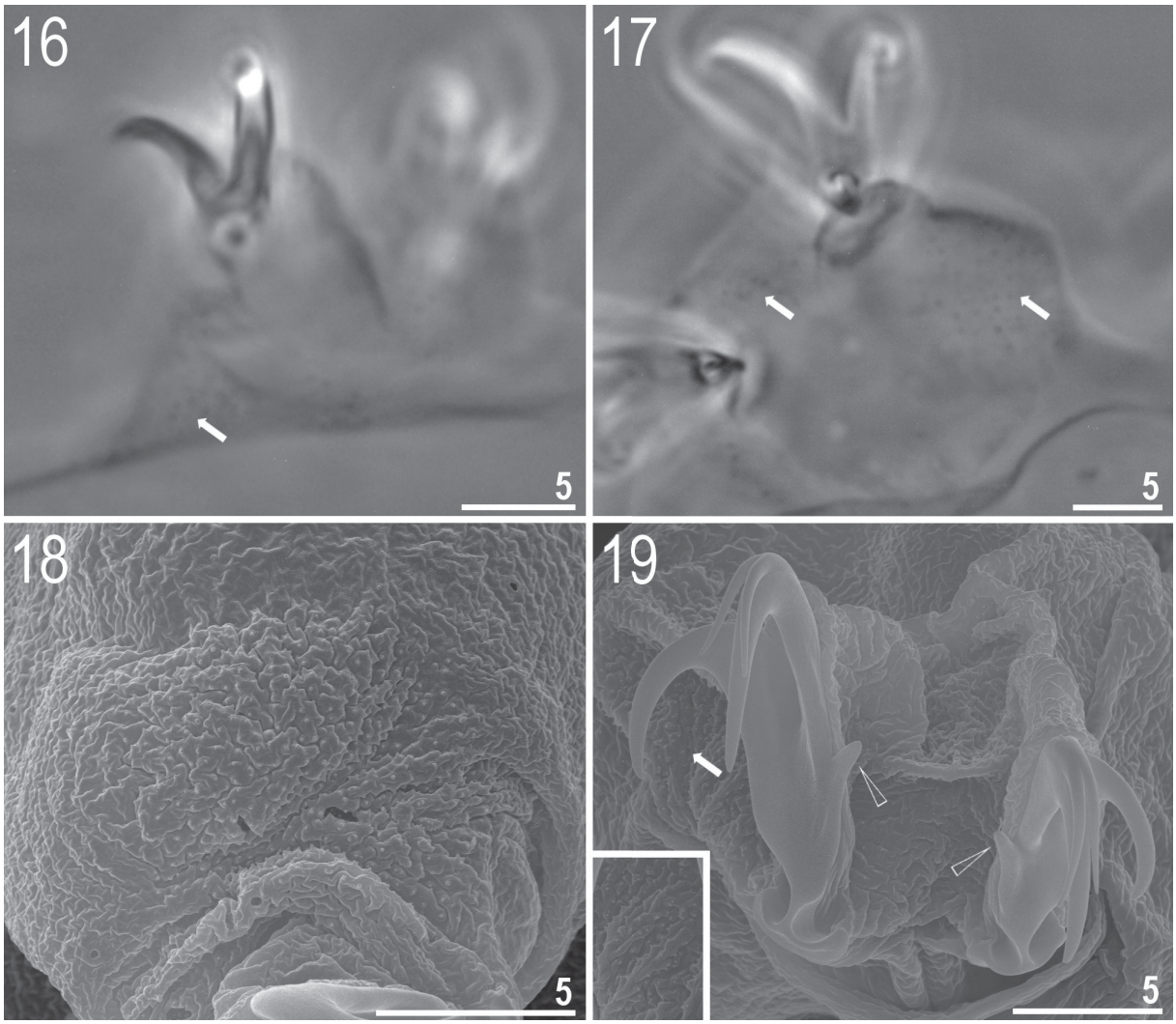

**Figs 10–15.** Claws. **10**. Claw II with smooth lunules (PCM, paratype). **11**. Claw IV (PCM, holotype). **12**. Indented lunules on claw IV (PCM, paratype). **13**. Claw I with smooth lunules (SEM, paratype). **14**. Claw IV with indented lunules (SEM, paratype). **15**. Aberrant claw IV (SEM, paratype). 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 (0.9–1.2 μm in *Ma. polypiformis* sp. nov. vs 0.3–0.5 μm in *Ma*. *pauliane*), 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 (0.5–0.8 μm in *Ma. polypiformis* sp. nov. vs

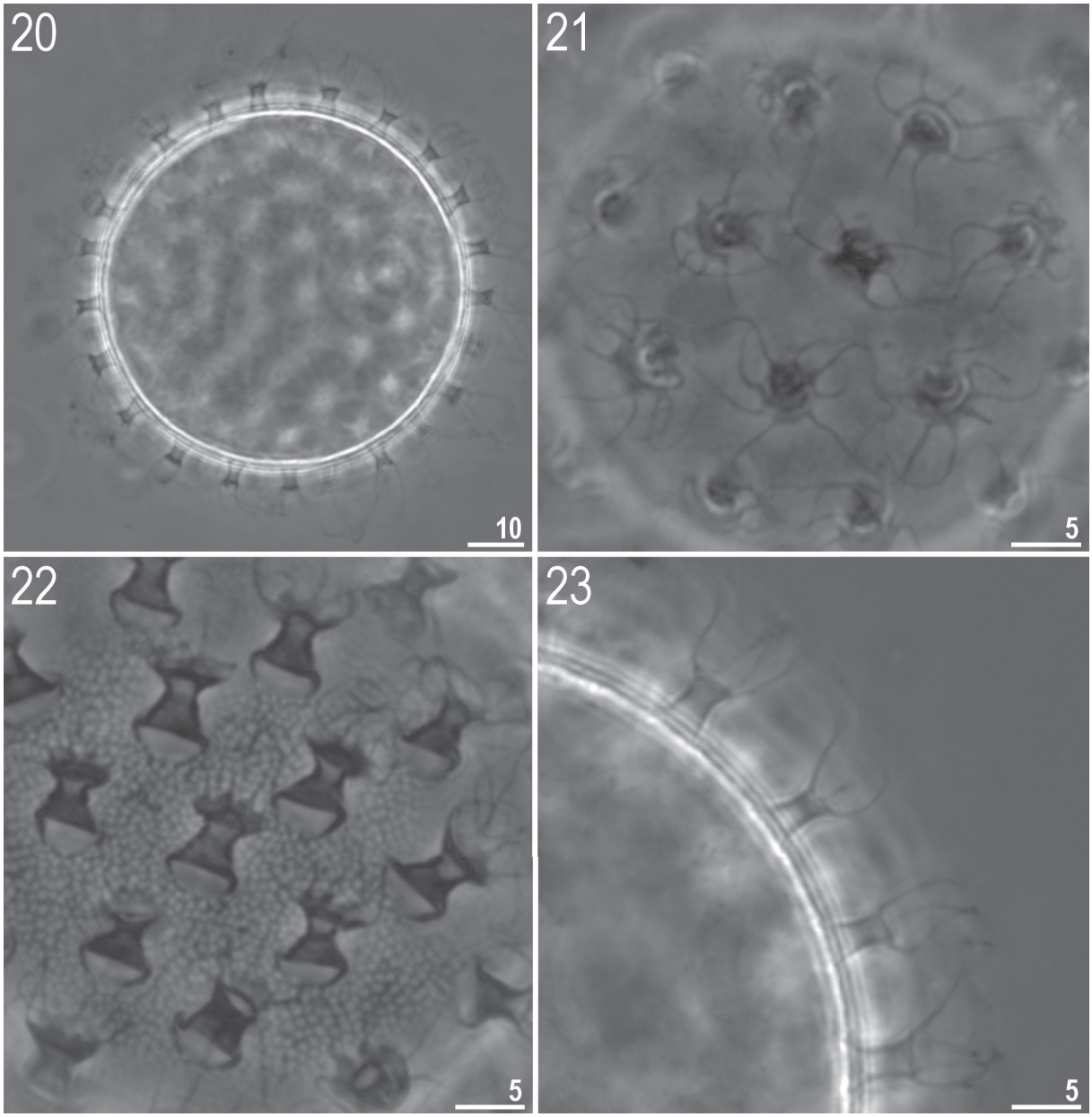



**Figs 16–19.** *Macrobiotus polypiformis* sp. nov., paratypes. **16**. Granulation on the II pair of legs, arrow (PCM). **17**. Granulation on IV pair of legs, arrow (PCM). **18**. Granulation on II pair of legs (SEM). **19**. Modified claw IV (arrowheads) and granulation on IV pair of legs, arrow (SEM).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



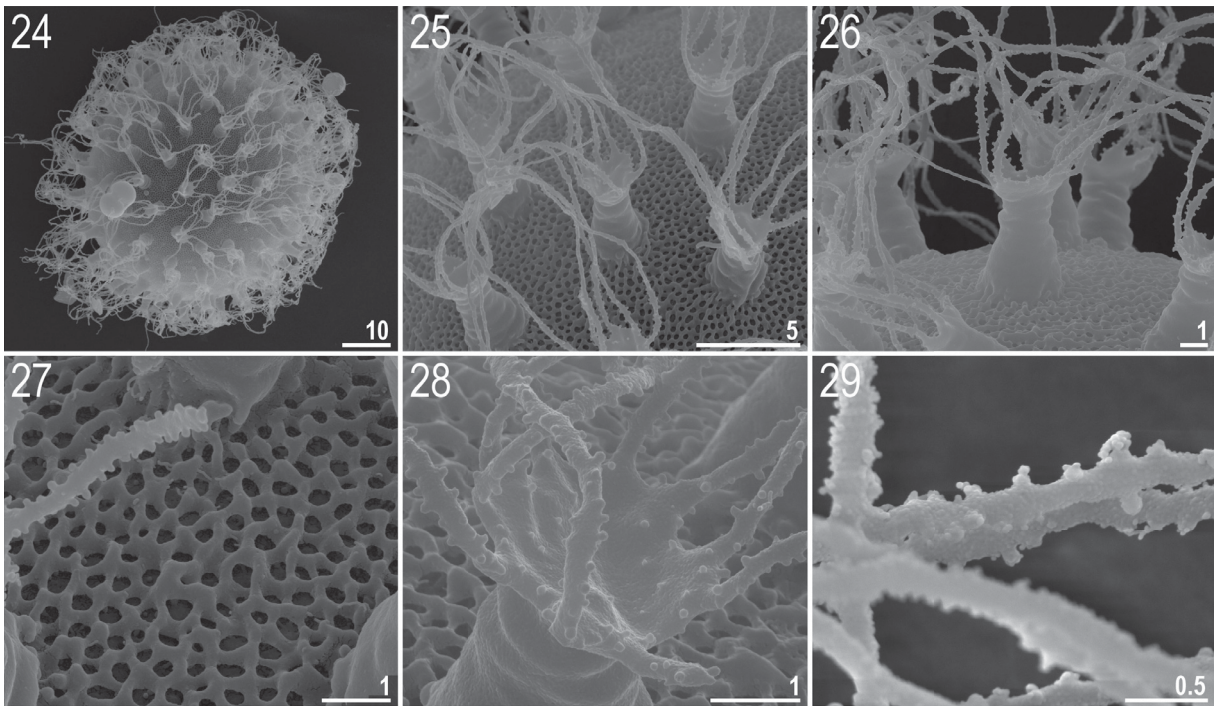

**Figs 20–23.** *Macrobiotus polypiformis* sp. nov. **20**. Chorion. **21**. Long, hair-like flexible filaments on terminal discs. **22**. Egg processes and the surface between egg processes with reticular design. **23**. Egg processes with filaments. All in PCM. Scale bars in μm.

*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 (Dastych 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. (Dastych 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 *Macrobiotus*, *Mesobiotus* or *Paramacrobiotus* 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 subanomalus* (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.

## Acknowledgements

The authors wish to thank Brian Blagden (Scottish Environment Protection Agency, UK) for his help in the English revision and for his suggestions on the manuscript. This work was partially supported by the *Prometeo Project* of the Secretariat for Higher Education, Science, Technology and Innovation of the Republic of Ecuador (grant to ŁK) and by the *Homing Plus* programme of the Foundation for Polish Science, co-funded by the European Union's Regional Development Fund (grant no. HP/2012-5/8 to Łukasz Michalczyk, Jagiellonian University, Poland). The research was partially carried out thanks to equipment purchased with the financial assistance of the European Regional Development Fund in the framework of the Polish Innovation Economy Operational Program (contract no. POIG.02.01.00-12-023/08 to the Jagiellonian University, Poland). Studies have been conducted in the framework of activities of BARg (Biodiversity and Astrobiology Research group at the Adam Mickiewicz University in Poznań, Poland). We also wish to thank the Ministry of Education, Youth and Sports of the Czech Republic (project numbers: LM2015078 and CZ.02.1.01/0.0/0.0/15_003/0000460) for financial support.

## References

Altschul S.F., Gish W., Miller W., Myers E.W. & Lipman D.J. 1990. Basic local alignment search tool. *Journal of Molecular Biology* 215: 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Bąkowski M., Roszkowska M., Gawlak M. & Kaczmarek Ł. 2016. *Macrobiotus naskreckii* sp. nov. (Eutardigrada: Macrobiotidae: *hufelandi* group) from Mozambique. *Annales Zoologici* 66 (2): 155–164.

Bertolani R. & Rebecchi L. 1993. A revision of the *Macrobiotus hufelandi* group (Tardigrada, Macrobiotidae), with some observations on the taxonomic characters of eutardigrades. *Zoologica Scripta* 22: 127–152. https://doi.org/10.1111/j.1463-6409.1993.tb00347.x

Bertolani R., Biserov V.I., Rebecchi L. & Cesari M. 2011. Taxonomy and biogeography of tardigrades using an integrated approach: new results on species of the *Macrobiotus hufelandi* group. *Invertebrate Zoology* 8 (1): 23–36.

Bertolani R., Guidetti R., Marchioro T., Altiero T., Rebecchi L. & Cesari M. 2014. Phylogeny of Eutardigrada: New molecular data and their morphological support lead to the identification of new evolutionary lineages. *Molecular Phylogenetics and Evolution* 76: 110–126. https://doi.org/10.1016/j.ympev.2014.03.006

Casquet J., Thebaud C. & Gillespie R.G. 2012. Chelex without boiling, a rapid and easy technique to obtain stable amplifiable DNA from small amounts of ethanol-stored spiders. *Molecular Ecology Resources* 12: 136–141. https://doi.org/10.1111/j.1755-0998.2011.03073.x

Cesari M., Bertolani R., Rebecchi L. & Guidetti R. 2009. DNA barcoding in Tardigrada: the first case study on *Macrobiotus macrocalix* Bertolani & Rebecchi 1993 (Eutardigrada, Macrobiotidae). *Molecular Ecology Resources* 9 (3): 699–706. https://doi.org/10.1111/j.1755-0998.2009.02538.x

Dastych H. 1980. *Niesporczaki (Tardigrada) Tatrzańskiego Parku Narodowego*. Monografie Fauny Polski 9, PWN, Krakow.

Dastych H. 1984. The Tardigrada from Antartica with description of several new species. *Acta Zoologica Cracoviensia* 27: 377–436.

Degma P. & Guidetti R. 2007. Notes to the current checklist of Tardigrada. *Zootaxa* 1579: 41–53.

Degma P., Bertolani R. & Guidetti R. 2009–2016. Actual checklist of Tardigrada species. (v. 31: 15 Dec. 2016). Available from www.tardigrada.modena.unimo.it/miscellanea/Actual%20checklist%20of%20Tardigrada.pdf [accessed 6 Apr. 2017].

De Oliveira Dias C. 1999. Morphological abnormalities of *Acartia lilljeborgi* (Copepoda, Crustacea) in the Espirito Santo Bay (E.S. Brazil). *Hydrobiologia* 394: 249–251. https://doi.org/10.1023/A:1003502624336

Elmoor-Loureiro L.M. 2004. Morphological abnormalities in the cladoceran *Ilyocryptus spinifer* (Apipucos Reservoir, Pernambuco State, Brazil). *Brazilian Journal of Biology* 64 (1): 53–58. https://doi.org/10.1590/S1519-69842004000100007

Folmer O., Black M., Hoeh W., Lutz R. & Vrijenhoek R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* 3: 294–299.

Fransozo A., Teixeira G.M., Gomes R.R., Silva J.C. & Bolla Jr. E.A. 2012. Ocorrência de anormalidades morfológicas externas em caranguejos marinhos (Decapoda, Brachyura) no litoral norte do estado de São Paulo. *Acta Scientiarum – Biological Sciences* 34 (1): 101–104.

Geslin E., Debenay J., Duleba W. & Bonetti C. 2002. Morphological abnormalities of foraminiferal tests in Brazilian environments: comparison between polluted and non-polluted areas. *Marine Micropaleontology* 45 (2): 151–168. https://doi.org/10.1016/S0377-8398(01)00042-1

Głowska E. & Skoracki M. 2009. Anomalous chaetotaxy in the quill mites *Torotrogla merulae* Skoracki *et al*., 2000 (Acari, Prostigmata, Syringophilidae). *Acta Parasitologica* 54 (4): 364–367. https://doi.org/10.2478/s11686-009-0046-y

Guidetti R. & Bertolani R. 2005. Tardigrade taxonomy: an updated check list of the taxa and a list of characters for their identification. *Zootaxa* 845: 1–46.

Guidetti R., Altiero T., Marchioro T., Sarzi Amadè L., Avdonina A.M., Bertolani R. & Rebecchi L. 2012. Form and function of the feeding apparatus in Eutardigrada (Tardigrada). *Zoomorphology* 131 (2): 127–148. https://doi.org/10.1007/s00435-012-0149-0

Guidetti R., Peluffo J.R., Rocha A.M., Cesari M. & Moly de Peluffo M.C. 2013. The morphological and molecular analyses of a new South American urban tardigrade offer new insights on the biological meaning of the *Macrobiotus hufelandi* group of species (Tardigrada: Macrobiotidae). *Journal of Natural History* 47: 2409–2426. https://doi.org/10.1080/00222933.2013.800610

Guil N. & Giribet G. 2012. A comprehensive molecular phylogeny of tardigrades - adding genes and taxa to a poorly resolved phylum-level phylogeny. *Cladistics* 28 (1): 21–49. https://doi.org/10.1111/j.1096-0031.2011.00364.x

Hall T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41: 95–98.

Hebert P.D.N., Cywinska A., Ball S.L. & de Waard J.R. 2003. Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London, Series B* 270: 313–322. https://doi.org/10.1098/rspb.2002.2218

Horning D.S., Schuster R.O. & Grigarick A.A. 1978. Tardigrada of New Zealand. *New Zealand Journal of Zoology* 5: 185–280. https://doi.org/10.1080/03014223.1978.10428316

Kaczmarek Ł., Goldyn B., Prokop Z.M. & Michalczyk Ł. 2011. New records of Tardigrada from Bulgaria with the description of *Macrobiotus binieki* sp. nov. (Eutardigrada: Macrobiotidae) and a key to the species of the *harmsworthi* group. *Zootaxa* 2781: 29–39.

Kaczmarek Ł., Jakubowska N. & Michalczyk Ł. 2012. Current knowledge on Turkish tardigrades with a description of *Milnesium beasleyi* sp. nov. (Eutardigrada: Apochela: Milnesiidae, the *granulatum* group). *Zootaxa* 3589: 49–64.

Kaczmarek Ł., Cytan J., Zawierucha K., Diduszko D. & Michalczyk Ł. 2014. Tardigrades from Peru (South America), with descriptions of three new species of Parachela. *Zootaxa* 3790 (2): 357–379. https://doi.org/10.11646/zootaxa.3790.2.5

Kaczmarek Ł., Michalczyk Ł. & McInnes S.J. 2015. Annotated zoogeography of non-marine Tardigrada. Part II: South America. *Zootaxa* 3923 (1): 1–107. https://doi.org/10.11646/zootaxa.3923.1.1

Kaczmarek Ł., Michalczyk Ł. & McInnes S.J. 2016. Annotated zoogeography of non-marine Tardigrada. Part III: North America and Greenland. *Zootaxa* 4203 (1): 001–249. https://doi.org/10.11646/zootaxa.4203.1.1

Londoño R., Daza A., Caicedo M., Quiroga S. & Kaczmarek Ł. 2015. The genus *Milnesium* (Eutardigrada: Milnesiidae) in the Sierra Nevada de Santa Marta (Colombia), with the description of *Milnesium kogui* sp. nov. *Zootaxa* 3955 (4): 561–568. https://doi.org/10.11646/zootaxa.3955.4.7

Marley N.J., McInnes S.J. & Sands C.J. 2011. Phylum Tardigrada: a re-evaluation of the Parachela. *Zootaxa* 2819: 51–64.

McInnes S.J. 1994. Zoogeographic distribution of terrestrial/freshwater tardigrades from current literature. *Journal of Natural History* 28: 257–352. https://doi.org/10.1080/00222939400770131

Méndez-de Daboín Y., Oviedo-Araújo M., González-Pérez A., Suárez-Hernández J., Sandoval C.M. & Cazorla D. 2015. Anomalías morfológicas en los dientes del cibario de *Lutzomyia evansi* (Diptera: Psychodidae) en el estado Trujillo, Venezuela. *Biomedica* 35 (2): 269–273.

Meriç E., Avşar N., Yokeş M.B., Tuğrul A.B., Bayari S., Özyurt N., Barut I.F., Balkis N., Uysal K. & Kam E. 2008. Morphological abnormalities in benthic foraminifers of the Antalya coast. *Micropaleontology* 54 (3–4): 241–276.

Michalczyk Ł. & Kaczmarek Ł. 2003. A description of the new tardigrade *Macrobiotus reinhardti* (Eutardigrada: Macrobiotidae, *harmsworthi* group) with some remarks on the oral cavity armature within the genus *Macrobiotus* Schultze. *Zootaxa* 331: 1–24.

Michalczyk Ł. & Kaczmarek Ł. 2013. The Tardigrada Register: a comprehensive online data repository for tardigrade taxonomy. *Journal of Limnology* 72 (S1): 175–181. https://doi.org/10.4081/jlimnol.2013.s1.e22

Michalczyk Ł., Wełnicz W., Frohme M. & Kaczmarek Ł. 2012. Redescriptions of three *Milnesium* Doyère, 1840 taxa (Tardigrada: Eutardigrada: Milnesiidae), including the nominal species for the genus. *Zootaxa* 3154: 1–20.

Miličić D., Pavković-Lučić S. & Lučić L. 2013. On some morphological abnormalities in adult fairy shrimp *Branchipus schaefferi* Fischer, 1834, from Serbia. *Archives of Biological Sciences* 65 (4): 1645–1650. https://doi.org/10.2298/ABS1304645M

Mironov S.V., Dabert J. & Dabert M. 2012. A new feather mite species of the genus *Proctophyllodes* Robin, 1877 (Astigmata: Proctophyllodidae) from the Long-tailed Tit *Aegithalos caudatus* (Passeriformes: Aegithalidae): morphological description with DNA barcode data. *Zootaxa* 3253: 54–61.

Morek W., Gąsiorek P., Stec D., Blagden B & Michalczyk Ł. 2016. Experimental taxonomy exposes ontogenetic variability and elucidates the taxonomic value of claw configuration in *Milnesium* Doyère, 1840 (Tardigrada: Eutardigrada: Apochela). *Contributions to Zoology* 85 (2): 173–200.

Nelson D.R., Guidetti R. & Rebecchi L. 2015. Chapter 17: Phylum Tardigrada. *In:* Thorp J. & Rogers D.C. (eds) *Ecology and General Biology. Vol. 1*: 347–380. Thorp and Covich's Freshwater Invertebrates (4th edition), Elsevier, Amsterdam.

Pilato G. 1981. Analisi di nuovi caratteri nello studio degli Eutardigradi. *Animalia* 8: 51–57.

Pilato G. & Binda M.G. 1996. Two new species and new records of *Macrobiotus* (Eutardigrada) from New Zealand. *New Zealand Journal of Zoology* 23: 375–379.

Pilato G. & Binda M.G. 2010. Definition of families, subfamilies, genera and subgenera of the Eutardigrada, and keys to their identification. *Zootaxa* 2404: 1–52.

Ramazzotti G. & Maucci W. 1983. *Il Phylum Tardigrada*. Memorie dell'Istituto Italiano di Idrobiologia, Pallanza 41, Istituto Italiano di Idrobiologia, Verbania.

Reinert J.F. 1999. Morphological abnormalities in species of the *quadrimaculatus* complex of *Anopheles* (Diptera: Culicidae). *Journal of the American Mosquito Control Association* 15 (1): 8–14.

Roszkowska M., Ostrowska M. & Kaczmarek Ł. 2015. The genus *Milnesium* Doyère, 1840 (Tardigrada) in South America with descriptions of two new species from Argentina and discussion of the feeding behaviour in the family Milnesiidae. *Zoological Studies* 54: 1–17. https://doi.org/10.1186/s40555-014-0082-7

Roszkowska M., Stec D., Ciobanu D.A. & Kaczmarek Ł. 2016. Tardigrades from Nahuel Huapi National Park (Argentina, South America) with descriptions of two new Macrobiotidae species. *Zootaxa* 4105 (3): 243–260. https://doi.org/10.11646/zootaxa.4105.3.2

Samir A.M. & El-Din A.B. 2001. Benthic foraminiferal assemblages and morphological abnormalities as pollution proxies in two Egyptian bays. *Marine Micropaleontology* 41 (3-4): 193–227. https://doi.org/10.1016/S0377-8398(00)00061-X

Sands C.J., Mcinnes S.J., Marley N.J., Goodall-Copestake W., Convey P. & Linse K. 2008. Phylum Tardigarda: an "individual" approach. *Cladistics* 24 (6): 861–871. https://doi.org/10.1111/j.1096-0031.2008.00219.x

Schill R.O., Forster F., Dandekar T. & Wolf N. 2010. Using compensatory base change analysis of internal transcribed spacer 2 secondary structures to identify three new species in *Paramacrobiotus* (Tardigrada). *Organisms Diversity & Evolution* 10 (4): 287–296. https://doi.org/10.1007/s13127-010-0025-z

Stec D., Smolak R., Kaczmarek Ł. & Michalczyk Ł. 2015. An integrative description of *Macrobiotus paulinae* sp. nov. (Tardigrada: Eutardigrada: Macrobiotidae: *hufelandi* group) from Kenya. *Zootaxa* 4052 (5): 501–526. https://doi.org/10.11646/zootaxa.4052.5.1

Stec D., Gąsiorek P., Morek W., Kosztyła P., Zawierucha K., Michno K., Kaczmarek Ł., Prokop Z.M. & Michalczyk Ł. 2016a. Estimating optimal sample size for tardigrade morphometry. *Zoological Journal of the Linnean Society* 178 (4): 776–784. https://doi.org/10.1111/zoj.12404

Stec D., Morek W., Gąsiorek P., Kaczmarek Ł. & Michalczyk Ł. 2016b. Determinants and taxonomic consequences of extreme egg shell variability in *Ramazzottius subanomalus* (Biserov, 1985) (Tardigrada). *Zootaxa* 4208 (2): 176–188. https://doi.org/10.11646/zootaxa.4208.2.5

St Louis R., Burningham L., Dowl A. & Grosberg M. 2009. *Lonely Planet Guide: Ecuador & the Galápagos Islands*. Lonely Planet, New York.

Tamura K., Dudley J., Nei M. & Kumar S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24: 1596–1599. https://doi.org/10.1093/molbev/msm092

Thompson J.D., Higgins D.G. & Gibson T.J. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positionspecific gap penalties and weight matrix choice. *Nucleic Acids Research* 22 (22): 4673–80.

Tumanov D.V. 2006. Five new species of the genus *Milnesium* (Tardigrada, Eutardigrada, Milnesiidae). *Zootaxa* 1122: 1–23.

Vicente F. & Bertolani R. 2013. Considerations on the taxonomy of the Phylum Tardigrada. *Zootaxa* 3626: 245–248. https://doi.org/10.11646/zootaxa.3626.2.2

Wełnicz W., Grohme M.A., Kaczmarek Ł., Schill R.O. & Frohme M. 2011. ITS-2 and 18S rRNA data from *Macrobiotus polonicus* and *Milnesium tardigradum* (Eutardigrada, Tardigrada). *Journal of Zoological Systematics and Evolutionary Research* 49 (S1): 34–39. https://doi.org/10.1111/j.1439-0469.2010.00595.x

White T.J., Bruns T., Lee S. & Taylor J. 1990. *PCR protocols: a guide to methods and application*: 315–322. Academic Press, San Diego, California.

Zagal C.J. 2008. Morphological abnormalities in the stauromedusa *Haliclystus auricula* (Cnidaria) and their possible causes. *Journal of the Marine Biological Association of the United Kingdom* 88 (2): 259–262. https://doi.org/10.1017/S0025315408000179

*Manuscript received: 3 June 2016*
*Manuscript accepted: 1 September 2016*
*Published on: 7 June 2017*
*Topic editor: Rudy Jocqué*
*Desk editor: Kristiaan Hoedemakers*