Research paper

An integrative description of *Minibiotus ioculator* sp. nov. from the Republic of South Africa with notes on *Minibiotus pentannulatus*

Londoño et al., 2017 (Tardigrada: Macrobiotidae)

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**Abstract**

The genus *Minibiotus* is morphologically diverse, which may suggest its polyphyletic character. However, scarce genetic data and often also the lack of detailed morphological data currently do not allow for the verification of the relationships within this genus. Here, for the very first time, we provide an integrative description of a new *Minibiotus* species, *Minibiotus ioculator* sp. nov. from the Republic of South Africa differs from other congeners mainly by egg ornamentation with processes on the egg shell that resemble the hat of a royal jester. We also provide new taxonomic data on *Minibiotus pentannulatus* based on a population newly found in Tanzania, which constitutes the first African record of this species originally described from South America. Our study involved both classical taxonomic methods, which include morphological and morphometric analyses conducted with the use of light and scanning electron microscopy, and genetic data in the form of DNA sequences of four markers (three nuclear: 18S rRNA, 28S rRNA, ITS-2, and one mitochondrial: COI). The results of this study allow a discussion of species composition within *Minibiotus* and question the validity of the current diagnosis of the genus.

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1. Introduction

Tardigrada are a phylum of microscopic invertebrates known also as water bears. Tardigrades are distributed globally and they inhabit various environments, from ocean depths to mountain peaks, and from polar caps to tropical forests (Nelson et al. 2015).

Up to date, about 1300 species have been described in the phylum (Guidetti & Bertolani 2005; Degma & Guidetti 2007; Degma et al. 2009–2019).

The genus *Minibiotus* R.O. Schuster, 1980 was erected fourty years ago by Schuster et al. (1980). Almost two decades later Claxton (1998) published a comprehensive revision of this cosmopolitan genus, which then encompassed 22 species and currently comprises 48 species (Degma et al. 2009–2019). However, only nine *Minibiotus* species have been reported from Africa so far, out of which five were described specifically from this continent. These are: *Minibiotus africanus* Binda & Pilato, 1995, *Minibiotus allani* (Murray, 1913), *Minibiotus crassidens* (Murray, 1907), *Minibiotus granatii* (Pardi, 1941), and *Minibiotus harrylewisi* Meyer & Hinton, 2009.

Currently, genetic data for the genus are very scarce. Specifically, there are only fourteen *Minibiotus* DNA sequences deposited in GenBank. Moreover, almost all of them are unidentified species or the entries are identified as “*Minibiotus intermedii* (*Plate, 1888*)” of which a certain identification is impossible due to the outdated and incomplete original description. In fact, there is only one named species, *Minibiotus gummiesoidai* Guil & Guidetti, 2005, which is associated with multiple (i.e. 18S rRNA, 28S rRNA and COI) sequences (Guil & Gribet 2012). The currently available DNA sequences suggest that *Minibiotus* is most likely polyphyletic and some species are closely related to *Paramacrobiotus* Guidetti et al., 2009, forming together one of the two major evolutionary lineages within Macrobiotidae (Bertolani et al. 2014). However, the extremely limited genetic data prevent any sound conclusions on the phylogenetic character and position of the genus *Minibiotus*. The putative polyphyly of *Minibiotus* is also suggested by the morphological heterogeneity of the genus, which comprises...
species with two and three macroplacoids in the pharynx, species with and without pores in the body cuticle, or species with egg processes enclosed within membrane and with naked processes (Stec et al. 2015). The broad morphological diagnosis combined with the small size of specimens which entails difficulties in the determination of some characters (e.g. peribuccal structures) sometimes result in erroneous assignments of species to the genus (e.g. see Stec et al. 2015 who transferred two Minibiotus species to the genus Macrobiotus).

In this paper, we provide the first description of a new Minibiotus species by means of integrative taxonomy. In addition to the description of Minibiotus loculator sp. nov. from the Republic of South Africa, we also present integrative data and amend the description of Minibiotus pentannulatus Londono, Daza, Lisi & Quiroga, 2017 based on a newly found population from Tanzania. The detailed morphological and morphometric data were obtained using light contrast and scanning electron microscopy. These data are associated with DNA sequences of four genetic markers standardly used in tardigrade taxonomy (the nuclear 18S rRNA, 28S rRNA, and ITS-2, and the mitochondrial COI).

2. Material and methods

2.1. Samples collection and processing

The lichen sample containing the new species was collected by Witold Morek and Bartomiej Szymacz on 7 September 2018 from the rock in forest in the Tradouw Pass located in Western Cape, South Africa (33°58′58.44″S, 20°42′17.88″E; 295 m asl). The lichen sample containing M. pentannulatus was collected by Thomas Pape on 16 August 2016 from branches of a bush, near the Mwanahama Peak in the Udzungwa Mts. National Park in Tanzania (7°49′25″S, 36°49′32″E; 2050 m asl). The latter sample contained also a new species of the Macrobiotus hufelandi group, which has been recently described as Macrobiotus papei Stec, Kristensen & Michalczyk, 2018.

The samples were examined for tardigrades using the protocol by Dastych (1980) with modifications described in detail in Stec et al. (2015). A total of 99 and 83 animals and 31 and 46 eggs of the two Minibiotus species were extracted from the South African and Tanzanian samples, respectively. In order to perform integrative taxonomic descriptions, the isolated animals and eggs were split into three groups for specific analyses: morphological analysis with phase and differential contrast light microscopy, morphological analysis with scanning electron microscopy, and DNA sequencing (for details see sections “Material examined” provided below for each description).

2.2. Microscopy and imaging

Specimens for light microscopy were mounted on microscope slides in a small drop of Hoyer’s medium and secured with a cover slip, following the protocol by Morek et al. (2016). Slides were examined under an Olympus BX53 light microscope with phase and Nomarski contrast (PCM and NCM, respectively), collectively termed as light contrast microscopy (LCM), associated with an Olympus DP74 digital camera. Immediately after mounting, the specimens in the medium slides where also checked under PCM for the presence of males and females in the studied population as the spermatozooa in testis and spermathecae are visible for several hours after mounting (Coughlan et al. 2019; Coughlan & Stec 2019). In order to obtain clean and extended specimens for SEM, tardigrades were processed according to the protocol by Stec et al. (2015). Bucco-pharyngeal apparatuses were extracted following the protocol of Eibye-Jacobsen (2001) as modified by Gasiorek et al. (2016). Specimens were examined under high vacuum in a Versa 3D DualBeam Scanning Electron Microscope (SEM) at the ATOMIN facility of the Jagiellonian University, Kraków, Poland. All figures were

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**Table 1**

| DNA fragment | Primer name | Primer direction | Primer sequence (5′-3′) | Source |
|--------------|-------------|------------------|------------------------|--------|
| 18S rRNA     | 18S_Tar_1Ff | forward          | AGGCAAAACCCGGAAAGGCTC  | Stec et al. (2017) |
|              | 18S_Tar_1Rr | reverse          | GCCGAGGTTCGCAAGTCGC    |        |
| 28S rRNA     | 28S_Eutar_F | forward          | ACCCCCTGACCTTATGATATAT | Gasiorek et al. (2018), Mironov et al. (2012) |
|              | Eutar_Rr   | reverse          | CTTGTCCTGCTGTTCAGAAC   |        |
| ITS-2        | LCO1490    | forward          | GTCTCTCCTTTATGATATGC   | Stec et al. (2018b) |
|              | Eutar_Rr   | reverse          | GCCGCCAGGCTCCACTCCTGG   |        |
| COI          | COI        | forward          | GTAAATATATGRTGDGCTC    |        |
|              | HCOoutout   | reverse          | TCCTCGGTATTGATATGC     | Fornier et al. (1994) |

**Table 2**

| DNA marker | Species                        | Accession number | Source               |
|------------|--------------------------------|------------------|----------------------|
| 18S rRNA   | M. loculator sp. nov.          | MT023998         | This study           |
|           | M. pentannulatus Londono et al., 2017 | MT023999       | This study           |
|           | M. gumerisindoi Guil & Guidetti, 2005 | FJ435748       | Guil & Giribet (2012) |
|           | M. intermedius group           | HQ604979-80     | Bertolani et al. (2014)|
|           | Minibiotus sp.                 | EU266932-4      | Sands et al. (2008)  |
| 28S rRNA   | M. loculator sp. nov.          | MT024041         | This study           |
|           | M. pentannulatus Londono et al., 2017 | MT024042-3     | This study           |
|           | M. gumerisindoi Guil & Guidetti, 2005 | FJ435761       | Guil & Giribet (2012) |
| ITS-2      | M. loculator sp. nov.          | MT024000         | This study           |
|           | M. pentannulatus Londono et al., 2017 | MT024061       | This study           |
| COI        | M. loculator sp. nov.          | MT023412         | This study           |
|           | M. pentannulatus Londono et al., 2017 | MT023413-4     | This study           |
|           | M. gumerisindoi Guil & Guidetti, 2005 | FJ435803       | Guil & Giribet (2012) |
assembled in Corel Photo-Paint X6, ver. 16.4.1.1281. For structures that could not be satisfactorily focused in a single light microscope photograph, a stack of 2–6 images were taken with an equidistance of ca. 0.2 μm and assembled manually into a single deep-focus image in Corel Photo-Paint X6, ver. 16.4.1.1281.

2.3. Morphometrics and morphological nomenclature

All measurements are given in micrometres (μm). Sample size was adjusted following recommendations by Stec et al. (2016). Structures were measured only if their orientation was suitable. Body length was measured from the anterior extremity to the end of the body, excluding the hind legs. The terminology used to describe oral cavity armature and egg shell morphology follows Michalczyk (2003) and Kaczmarek (2013). The type of buccal apparatus and claws are given according to Pilato & Binda (2010). Macroplacid length sequence is given according to Kaczmarek et al. (2014). Buccal tube length and the level of the stylet support insertion point were measured according to Pilato (1981). The pt index is the ratio of the length of a given structure to the length of the buccal tube expressed as a percentage (Pilato 1981). All other measurements and nomenclature follow Kaczmarek & Michalczyk (2017). Morphometric data were handled using the “Paracela” ver. 1.7 template available from the Tardigrada Register (Michalczyk & Kaczmarek 2013). Raw morphometric data for each analysed species are provided as supplementary materials (SM.01 and SM.02). Data underlying the description of the new species are also deposited in Tardigrada Register under www.tardigrada.net/register/0063.htm. Tardigrade taxonomy follows Guil et al. (2019).

2.4. Comparative material

To identify the Tanzanian population, beside the original description by Londoño et al. (2017), we also used microphotographs of a M. pentannullatus paratype kindly sent to us by Rosana Londoño and Anisheth Daza (Universidad del Magdalena, Colombia).

2.5. Genotyping

Individual DNA extractions were made from animals and/or eggs following a modified protocol by Casquet et al. (2012). Each specimen was placed individually in a 1.5 ml Eppendorf micro-centrifuge tube, in 45 μl of a 3% suspension of 75–150 μm wet bead size Chelex® 100 resin (Bio-Rad) in ddH2O with addition of 3.0 μl Proteinase K (A&A Biotechnology) and incubated at 56°C for 2 h followed by 7 min at 95°C. DNA was purified using a MasterPure Extracton Kit (Epicentre). Sequencing reactions and amplicon purification were made as described by Stec et al. (2016). Each specimen was sequenced in both directions using primers for mtcox1 (forward: 5′-CGAGATCAGAAGCTGCGCC-3′, reverse: 5′-TCTCTTGGTCTGCATCCA-3′) and mtcytc (forward: 5′-GATTTCCTTATTCTGGGGG-3′, reverse: 5′-TCTCCTACATATCCAAAA-3′). These primers were chosen based on the presence of suitable sequences in GenBank. Sequence data were deposited in GenBank under accession numbers MA050047 and MA050048.

Table 3

| CHARACTER          | N            | RANGE                 | MEAN       | SD        | Holotype |
|--------------------|--------------|-----------------------|------------|-----------|----------|
|                    | μm           | pt                    | μm pt      | μm pt     |          |
| Body length        | 30           | 197–371               | 891–1267   | 313 1121  | 43 85    | 312 1106 |
| Buccopharyngeal tube |             |                       |            |           |          |
| Buccal tube length | 30           | 21.8–30.8             | −          | 27.8 −    | 2.3 −    | 28.2 −   |
| Stylet support insertion point | 30 13.4–19.1 | 61.1–62.8            | 17.3 62.2  | 1.5 0.5   | 17.7 62.8|
| Buccal tube external width | 30 1.8–2.4 | 6.4–8.6              | 2.1 7.7   | 0.2 0.5   | 2.1 7.4  |
| Buccal tube internal width | 30 0.7–1.6 | 2.8–6.0              | 1.0 3.8   | 0.2 0.6   | 0.8 2.8  |
| Ventral lamina length | 30 9.3–13.9 | 41.9–49.5            | 12.4 44.6 | 1.1 1.8   | 12.5 44.3|
| Macroploaid lengths |             |                       |            |           |          |
| Macroploaid 1      | 30           | 2.0–3.4               | 8.9–12.4   | 2.9 10.5  | 0.3 0.8  | 3.0 10.6 |
| Macroploaid 2      | 30           | 1.5–2.4               | 6.4–8.4    | 2.0 7.3   | 0.2 0.5  | 2.2 7.8  |
| Macroploaid 3      | 30           | 1.8–3.0               | 7.8–10.2   | 2.5 8.9   | 0.2 0.6  | 2.3 8.2  |
| Microplaoaid       | 30           | 0.7–1.6               | 2.9–5.3    | 1.1 3.9   | 0.2 0.6  | 1.0 3.5  |
| Macroploaid row    | 30           | 6.3–10.4              | 27.0–35.3  | 8.8 31.6  | 0.9 1.7  | 8.9 31.6 |
| Placoid row        | 30           | 7.2–12.4              | 32.1–41.4  | 10.2 36.8 | 1.1 2.1  | 10.4 36.9|

* pt values in tardigrade measurements are always provided with italics.

Table 4

| CHARACTER          | N            | RANGE                 | MEAN       | SD        |
|--------------------|--------------|-----------------------|------------|-----------|
|                    | μm           | pt                    | μm pt      |           |
| Egg full diameter  | 14           | 66.2–81.1             | 73.6       | 4.3       |
| Egg bare diameter  | 14           | 54.2–70.1             | 61.9       | 4.1       |
| Process height     | 42           | 4.5–7.9               | 5.6        | 0.9       |
| Process base width | 42           | 2.1–4.2               | 2.9        | 0.4       |
| Process base/height ratio | 42 41%–69% | 53% 7%       |
| Inter-process distance | 42 1.2–3.0 | 2.1 0.4                 |
| Number of processes on the egg circumference | 14 30–32 | 31.3 0.8 |

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20–30 min in thermomixer with constant 500 rpm. Then, tubes were incubated at 70 °C for 10 min and after cooling to room temperature, the supernatant was transferred to new 1.5 ml tubes and stored in −20 °C. Before the extraction, specimens were mounted in water slides and check under microscope to confirm their identification. We sequenced four DNA fragments: the small ribosome subunit (18S rRNA, nDNA), the large ribosome subunit (28S rRNA, nDNA), the internal transcribed spacer (ITS-2, nDNA), and the cytochrome oxidase subunit I (COI, mtDNA). All fragments were amplified using the primers listed in Table 1. For every PCR reaction, the solution contained 12.25 μl ddH2O, 2 μl 10X DreamTaq Green Buffer (Thermo Scientific™), 0.8 μl 10 mM dNTPs, 0.8 μl 10 μM forward primer, 0.8 μl 10 μM reverse primer, 0.15 μl DreamTaq DNA Polymerase (5U/μl; Thermo Scientific™) and 3.2 μl of genomic DNA extract. The PCR profile for amplification of 28S rRNA, ITS-2 and COI was as follows: 5 min initial denaturation at 95 °C, followed by 30 s denaturation at 95 °C, 90 s annealing at 51 °C, 60 s elongation at 72 °C for 35 cycles and 10 min of final elongation at 72 °C. For amplification of 18S rRNA the same profile was used with the exception of annealing temperature which was increased to 55 °C for 5 s, and elongation at 60 °C for 4 min. 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) and submitted to GenBank (for the accession numbers please see Table 2).

### 2.6. Comparative genetic analysis

For molecular comparisons, all published sequences of the four abovementioned markers for species of the genus Minibiotus were downloaded from GenBank (Table 2) except single 18S rRNA and annealing at 55 °C for 5 s, and elongation at 60 °C for 4 min. 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) and submitted to GenBank (for the accession numbers please see Table 2).

### Table 5

| CHARACTER               | N   | RANGE               | MEAN | SD  |
|-------------------------|-----|---------------------|------|-----|
| Body length             | 30  | 198–279             | 244  | 928 |
| Buccopharyngeal tube    | 30  | 24.3–27.8           | 26.3 | –   |
| Buccal tube length      | 30  | 146.9–169           | 159  | 603 |
| Buccal tube external width| 30 | 1.7–2.2             | 2.0  | 7.7 |
| Buccal tube internal width | 30 | 0.8–1.2             | 1.0  | 3.9 |
| Ventral lamina length   | 29  | 11.5–14.1           | 12.6 | 48.0|
| Placoid lengths         |     |                     |      |     |
| Macroplacoid 1          | 30  | 1.6–2.6             | 2.1  | 8.0 |
| Macroplacoid 2          | 30  | 1.3–2.0             | 1.7  | 6.3 |
| Macroplacoid 3          | 30  | 1.3–2.1             | 1.7  | 6.4 |
| Microplacoid            | 30  | 0.5–1.1             | 0.8  | 2.9 |
| Macroplacoid row        | 30  | 6.1–8.1             | 7.1  | 27.1|
| Placoid row             | 30  | 7.4–10.1            | 8.4  | 32.1|
| Claw 1 lengths          |     |                     |      |     |
| External primary branch | 24  | 4.8–6.3             | 5.5  | 21.1|
| External secondary branch| 14 | 3.4–5.0             | 4.3  | 16.3|
| Internal primary branch | 23  | 4.7–5.7             | 5.2  | 19.9|
| Internal secondary branch| 13 | 3.4–4.3             | 4.0  | 15.0|
| Claw 2 lengths          |     |                     |      |     |
| External primary branch | 24  | 5.1–6.2             | 5.7  | 21.6|
| External secondary branch| 19 | 4.1–4.9             | 4.5  | 16.9|
| Internal primary branch | 23  | 4.6–6.0             | 5.4  | 20.6|
| Internal secondary branch| 17 | 3.5–4.7             | 4.1  | 15.7|
| Claw 3 lengths          |     |                     |      |     |
| External primary branch | 24  | 5.4–6.3             | 5.9  | 22.4|
| External secondary branch| 17 | 4.1–4.9             | 4.5  | 17.2|
| Internal primary branch | 24  | 5.1–6.2             | 5.6  | 21.2|
| Internal secondary branch| 15 | 3.8–4.5             | 4.2  | 16.1|
| Claw 4 lengths          |     |                     |      |     |
| Anterior primary branch | 26  | 5.8–7.4             | 6.6  | 24.9|
| Anterior secondary branch| 15 | 4.6–6.0             | 5.2  | 19.5|
| Posterior primary branch| 26  | 6.1–7.7             | 7.0  | 26.4|
| Posterior secondary branch| 17 | 4.8–6.1             | 5.4  | 20.4|

### Table 6

| CHARACTER            | N   | RANGE         | MEAN  |
|----------------------|-----|---------------|-------|
| Diameter of egg without processes | 30  | 50.2–65.4      | 58.6  |
| Diameter of egg with processes     | 30  | 60.8–77.0      | 69.1  |
| Process height                 | 90  | 2.2–6.8       | 4.6   |
| Process base width           | 90  | 2.6–5.4       | 3.8   |
| Process base/height ratio    | 90  | 49%–214%      | 87%   |
| Distance between processes  | 90  | 0.9–2.4       | 1.7   |
| Number of processes on the egg circumference | 30  | 30–36         | 32.3  |

### Table 7

Measurements [in μm] of selected morphological structures of individuals of Minibiotus pentannulatus Londério, Daza, Lisi & Quiroga, 2017 from Tanzania, mounted in Hoyer’s medium (N—number of specimens/structures measured; RANGE refers to the smallest and the largest structure among all measured specimens; SD—standard deviation).
Fig. 1. *Minibiotus ioculator* sp. nov. – habitus. (A) Dorso-ventral projection (holotype, Hoyer’s medium, PCM). Scale bars in μm.

Fig. 2. *Minibiotus ioculator* sp. nov. – cuticular structures on legs. (A–B) External granulation on leg III seen in PCM (A) and SEM (B) (paratypes). (C–D) Internal granulation on leg III and a cuticular bulge resembling a pulvinus seen in PCM (C) and SEM (D) (paratypes). (E–F) Granulation on leg IV seen in PCM (E) and SEM (F) (paratypes). Filled, flat arrowheads indicate the cuticular bulge. Scale bars in μm.
28S rRNA sequences (MH079468 and MH079492) published by Guil et al. (2019) as they represent a non-homologous fragment. The sequences were aligned using the default settings (in the case of ITS-2 and COI) and the QeINSI method (in the case of ribosomal markers: 18S rRNA, 28S rRNA) of MAFFT version 7 (Katoh et al. 2002; Katoh & Toh 2008) and manually checked against non-conservative alignments in BioEdit. Then, the aligned sequences were trimmed to: 760 (18S rRNA), 687 (28S rRNA), 437 (ITS-2), 620 (COI) bp. All COI sequences were translated into protein sequences in MEGA7 version 7.0 (Kumar et al. 2016) to check against pseudogenes. Uncorrected pairwise distances were calculated using MEGA7 and are provided as supplementary materials (SM.03).

3. Results

3.1. Taxonomic account of the new species

**Phylum:** Tardigrada Doyère 1840.

**Class:** Eutardigrada Richters 1926.

**Order:** Macrobiotoidea Guil et al. 2019.

**Family:** Macrobiotidae Thulin 1928.

**Genus:** Minibiotus R.O. Schuster, 1980 (in Schuster et al. 1980).

3.2. Minibiotus ioculator sp. nov.

(Tables 3 and 4, Figs. 1–8).

3.2.1. Material examined

99 animals (including 4 simplex), and 31 eggs. Specimens mounted on microscope slides in Hoyer’s medium (84 animals + 21 eggs), fixed on SEM stubs (10 + 10 + 2 buccal apparatuses), and processed for DNA sequencing (3 + 0).

3.2.2. Type locality

33°58′58.44″S, 20°42′17.88″E; 295 m asl: Republic of South Africa: Tradouw Pass located in Western Cape; lichen on rock in the forest; coll. 7 September 2018 by Witold Morek and Bartłomiej Surmacz.

3.2.3. Type depositories

Holotype (slide ZA.274.23 with 10 paratypes) and 68 paratypes (slides: ZA.274.* where the asterisk can be substituted by any of the following numbers 19, 22, 25–26; SEM stub: 18.14) and 23 eggs (slides: ZA.274.*: 20–21; SEM stub: 18.14) are deposited at the Institute of Zoology and Biomedical Research, Jagiellonian University, Gronostajowa 9, 30-387, Kraków, Poland and 14 paratypes (slide: ZA.274.*: 24) and 8 eggs (slides: ZA.274.*: 18) are deposited in the Natural History Museum of Denmark, University of Copenhagen, Universitetsparken 15, DK-2100 Copenhagen Ø, Denmark.

3.2.4. Etymology

The name refers to morphology of processes on the egg shell which resembles a hat of a royal jester. From Latin “jester” = “ioculator”.

3.2.5. Description of the new species

3.2.5.1. Animals (measurements and statistics in Table 3). Body whitish but transparent after fixation in Hoyer’s medium (Fig. 1A). Eyes present in live animals as well as in specimens mounted in Hoyer’s medium. Body cuticle smooth without pores and granulation, however clearly visible patches of big and dense granulation on all legs present (Fig. 2A–F). The granulation on legs I–III consists of two wide patches on the external and internal leg surface which are joined by narrow band of granulation situated on the anterior leg surface (Fig. 2A–D and 3A, C). The granulation on legs IV consists of a...
continuous and uniform patch which covers lateral and dorsal leg surfaces (Fig. 2E and F and 3B, D). A cuticular bulge/fold (pulvinus) is present on the internal surface of legs I–III (Fig. 2C and D).

Claws slender, of the *hufelandi* type (Fig. 3A–D). Primary branches with distinct accessory points, a common tract, and with an evident stalk connecting the claw to the lunula (Fig. 3A–D). Lunulae smooth on all legs (Fig. 3A–D). Cuticular bars under claws absent. Double muscle attachments faintly marked under LCM but clearly visible under SEM (Fig. 3A, C).

Mouth antero-ventral followed by ten peribuccal papulae (Fig. 4A–C; but see also Discussion). Bucco-pharyngeal apparatus of the *Minibiotus* type (Figs. 4A and 5A–C) with an anterior and a

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Fig. 4. *Minibiotus ioculator* sp. nov. — buccal apparatus and the oral cavity armature. (A) Dorsal view of the entire buccal apparatus (PCM), lower and upper inserts show ventral views of the oral cavity armature (PCM) and macroplacoids (NCM), respectively (all holotype). (B) Lateral view of the entire buccal apparatus (PCM) with the anterior and the posterior bend of the buccal tube. (C–D) The oral cavity armature of a single paratype seen in SEM from different angles showing dorsal (B) and ventral (C) views. Filled indented arrowheads indicate teeth of the first band, empty flat arrowheads indicate teeth of the second band whereas filled flat arrowheads indicate the third band of teeth, arrows indicate the anterior and the posterior bend of the buccal tube. Scale bars in μm.
3.2.5.2. Eggs (measurements and statistics in Table 4). Laid freely, white, spherical or slightly ovoid (Fig. 6A and B and 7A). The surface between processes smooth with depressions between processes faintly visible in LCM and clearly visible in SEM (Fig. 6B–F and 7A–F). In SEM, these depressions are perforated by micropores (Fig. 7B and C). However, depressions with micropores can sometimes be not visible due to particles of dirt/mucus which sometimes accumulate within the depressions (Fig. 7D–F). Processes are conical with a slender trunk and with apex often split into a few apices (Fig. 6C–F and 7D–F). The most common and characteristic appearance is a bifurcated process which resembles a hat of a medieval European royal jester (Fig. 6C–F and 7D). Process apices are covered by granules which are faintly visible in LCM but are clearly identifiable in SEM (Fig. 6C–F and 7D–F). Only occasionally, singular bubble-like structures can be seen inside the terminal portion of the processes (Fig. 6C–F). Sometimes, under LCM, the margins of processes bases seem to be serrated and surrounded by a crown of small thickenings (Fig. 6C–F) which are most probably internal strengthening structures stabilising the processes within the chorion as in other Macrobiotidae species (e.g. see Michalczyn & Kaczmarek 2003). In SEM, these structures are not visible in intact eggs (Fig. 7D–F).

3.2.5.3. Reproduction. The new species is dioecious. Although no spermathecae filled with sperm have been found in gravid females on the freshly prepared slides, the testis in males, filled with spermatozoa, was clearly visible under LCM up to 24 h after mounting in Hoyer’s medium (Fig. 8A). The new species does not exhibit sexual dimorphism such as lateral gibbosities on legs IV in males.

3.2.5.4. Phenotypic differential diagnosis. By smooth body cuticle without granulation and pores as well as by eggs with conical processes, the new species is similar to the following six Minibiotus species: M. allani (Murray, 1913), M. crassidens (Murray, 1907), Minibiotus aquatilis Claxton, 1998, Minibiotus hispidus Claxton, 1998, Minibiotus maculartus Pilato & Claxton, 1988, Minibiotus milleri Claxton, 1998. However, it differs specifically from:

- M. allani, reported only from the type locality in Kenya (Murray 1913), by: the presence of eyes (eyes absent in M. allani), the presence of granulation on all legs (granulation absent or not visible under light microscope in M. allani) and a different egg processes morphology (egg processes with slender trunks and endings split into stout arms with the most common and characteristic being division into two often curved arms which resemble a hat of a royal jester in the new species vs. egg processes with stouter trunks and endings split into several thin, flexible filaments in M. allani).
- M. crassidens, reported from five African countries: Angola (da Cunha & do Nascimento 1964), Democratic Republic of Congo (Teunissen 1938), Kenya (Murray 1913), Republic of South Africa (Murray 1907, 1913), and Uganda (Murray 1913), by: the presence of eyes (eyes absent in M. crassidens) and a different egg processes morphology (egg processes with slender trunks and endings split into stout arms with the most common and
characteristic being division into two often curved arms which resemble a hat of a royal jester in the new species vs. egg processes with undivided ending which is elongated into flexible one long flexible filament in *M. crassidens* and a less dense distribution of process on the egg surface (the processes densely distributed, almost in contact with each other so that the egg surface between processes is hardly visible in *M. crassidens*);

- *M. aquatilis*, reported from several localities in Australia and Tasmania (Claxton 1998), by: the presence of smooth lunules IV (lunules IV dentate in *M. aquatilis*), a different morphology of egg processes (egg processes with slender trunks and endings split into stout arms with the most common and characteristic being division into two often curved arms which resemble a hat of a royal jester in the new species vs. egg processes long with slender trunks elongated usually into one and only sometimes several flexible, hair-like filaments often with one line of small bauble-like structures visible inside in *M. aquatilis*), a different morphology of egg surface between processes (smooth surface with depressions faintly visible under LCM vs. the surface between processes covered by large dark dots arrange with single line around processes in *M. aquatilis*), and by shorter egg processes (4.5–7.9 μm in the new species vs. 11.0–12.5 μm in *M. aquatilis*);

- *M. hispidus*, reported only from Australia and New Zealand (Claxton 1998), by: a different morphology of egg processes (egg processes with slender trunks and endings split into stout arms with the most common and characteristic being division into two often curved arms which resemble a hat of a royal jester in the new species vs. egg processes in shape of small cones with greatly elongated, undivided endings in *M. hispidus*), a different morphology of egg surface between processes (smooth surface with depressions faintly visible under LCM vs. the surface between processes covered by small pores uniform in size with a distinct ring of pores surrounding each process in *M. hispidus*), and by a smaller number of process on the egg circumference (30–32 in the new species vs. 48 in *M. hispidus*);

- *M. maculartus*, reported from several localities in Australia and one in New Zealand (Pilato & Claxton 1988; Claxton 1998), by: the presence of smooth lunules IV (lunules IV dentate in
Fig. 7. *Minibiotus ioculator* sp. nov. – egg chorion seen in SEM. (A) Entire egg. (B) Details of the egg surface. (C) Magnification of a depression on the egg surface between the processes. (D–F) Details of the processes. Filled flat arrowheads indicate depressions present on the egg surface between the processes. Please note that on A and D–F the depressions are covered by dirt. Scale bars in μm.

Fig. 8. *Minibiotus ioculator* sp. nov. – reproduction. (A) Male testis (indicated by the arrow) seen in PCM, with visible spermatozoa in a male freshly mounted in Hoyer's medium. Scale bars in μm.
M. maculartus), a different morphology of egg processes (egg processes with slender trunks and endings split into stout arms with the most common and characteristic being division into two often curved arms which resemble a hat of a royal jester in the new species vs. wide conical processes with pointed apices with 8–9 longitudinal ridges extending from the processes base and joing the processes to the egg surface in M. maculartus), a different morphology of egg surface between processes (smooth surface with depressions faintly visible under LCM vs. the surface covered by irregularly distributed dark dots irregular in size and shape in M. maculartus), a shorter egg processes (4.5–7.9 μm in the new species vs. all processes about 11 μm in M. maculartus), a narrower processes bases (2.1–4.2 μm in the new species vs. all processes bases about 11 μm in M. maculartus), and by a higher number of process on the egg circumference (30–32 in the new species vs. 20–30 in M. milleri).

3.2.5.5. Genetic differential diagnosis. The ranges of uncorrected genetic p-distances between the new species and the few Minibiotus species, for which sequences are available from GenBank, are as follows:

- **18S rRNA**: 2.0–4.5% (3.7% on average), with the most similar being M. pentannulatus from Tanzania (MT023999) and the least similar being unidentified Minibiotus species from England (EU266934);

- **28S rRNA**: comparison with only two species: 7.2 and 7.3% difference with M. pentannulatus from Tanzania (MT024042 and MT024043, respectively) and 10.1% difference with M. gumersindoi from Spain (FJ435761);

- **ITS-2**: comparison with only one species: 19.2% difference with M. pentannulatus from Tanzania (MT024001);

- **COI**: comparison with only two species: 21.1% difference with M. pentannulatus from Tanzania (MT023413 and MT023414) and 23.9% difference with M. gumersindoi from Spain (FJ435803).

3.3. Minibiotus pentannulatus Londoño, Daza, Lisi & Quiroga, 2017

The population of Minibiotus species found in Tanzania and examined in this study was identified as M. pentannulatus based on the original description and new LCM microphotographs of type material. Data collected for this population allowed to amend the species description by providing more accurate morphological details from LCM and SEM as well as DNA barcodes for molecular species identification.
3.3.1. Material examined

83 animals (including 6 simplex), and 46 eggs. Specimens mounted on microscope slides in Hoyer’s medium (60 animals + 34 eggs), fixed on SEM stubs (20 + 9), and processed for DNA sequencing (3 + 3).

3.3.2. Locality

7°49’25”S, 36°49’32”E, 2050 m asl: Tanzania: Udzungwa Mts. National Park near the Mwanihana Peak; lichen from branches of a bush; coll. on 16 August 2016 by Thomas Pape.

3.3.3. Material depositories

69 animals (slides: TZ.027.* where the asterisk can be substituted by any of the following numbers 23, 25, 28, 30–31; SEM stubs: 14.02, 19.20) and 36 eggs (slides: TZ.027.*: 32–35; SEM stub: 14.02) are deposited at the Institute of Zoology and Biomedical Research, Jagiellonian University, Gronostajowa 9, 30-387, Kraków, Poland and 11 animals (slides: TZ.027.*: 26–27) and 7 eggs (slide: TZ.027.*: 36) are deposited in the Natural History Museum of Denmark, University of Copenhagen, Universitetsparken 15, DK-2100 Copenhagen Ø, Denmark.

3.3.4. Amended description

3.3.4.1. Animals (measurements and statistics in Table 5). Both types of cuticular pores (star-shaped and circular) reported in the original description are also obvious in SEM (Fig. 9A–D). The granulation on legs I–III was overlooked in the original description. In both populations the granulation is present on the external surface of legs I–III as a single small patch well visible in LCM (Fig. 10A–F) and in SEM (Fig. 11A, C). Moreover, a clear pattern of pore arrangement on legs I–III is also always present with one smaller star-shaped pore present in the centre of granulation patch and bigger star-shaped pore present above (Fig. 10A–F). The SEM analysis additionally confirmed this pattern revealing at the same time also a pulvinus on the internal surface of legs I–III (Fig. 11A–D). Under SEM, the granulation on all legs consist of microgranule aggregations being the most obvious on legs IV (Fig. 11A, C, E–F). The SEM analysis also confirmed that lunules on all legs are smooth (Fig. 11E and F). Bucco-pharyngeal apparatus of the Minibiotus type with the anterior and the posterior bend clearly visible in laterally positioned specimens under LCM (Fig. 12A). A cuticular fold with a pore in the centre is present just above the mouth opening and visible well under LCM (only in laterally positioned specimens) and under SEM (Fig. 12A and B). Mouth antero-ventral followed by ten short peribuccal papulae (Fig. 12B–D; but see also Discussion). As stated in the original description, the oral cavity armature is invisible under LCM. However, the SEM analysis confirmed the presence of teeth in the oral cavity. The oral cavity armature comprises three bands of teeth, with the first band being situated at the base of peribuccal lamellae and composed of a single row of small cone-shaped teeth fused to form a continuous, slightly serrated ring ridge around the oral cavity (Fig. 12C). The second band of teeth comprises one row of globular-shaped teeth with some smaller cone-shaped teeth distributed unevenly on the ring fold (Fig. 12C and D). The teeth of the third band are located within the posterior portion of the oral cavity, between the second band of teeth and the buccal tube.
opening (Fig. 12C), but only the dorsal portion of the third band of teeth is present (Fig. 12C and D). This portion is composed of three evidently separated, sharp cone-shaped teeth (Fig. 12C).

3.3.4.2. Eggs (measurements and statistics in Table 6). In the original description, the morphology of the egg ornamentation was reported based on a single egg. The egg shell ornamentation in the Tanzanian population conforms to that in the original description (Fig. 13A–F). The low sample size in the original description led Londoñ~o et al. (2017) to assume that egg processes always exhibit 5 latitudinal annulations, but the analysis of a larger number of eggs showed that number of annulation on egg process varies from 4 to 6 (Fig. 13A–F and 14A–F). Under SEM, the annulations are seen as a laminal rings with serrated/granulated margins surrounding the processes (Fig. 14A–F). Both LCM and SEM analysis confirmed that the chorion surface between the process is smooth, however we noted also the presence of internal strengthening thickenings at the processes bases which stabilise them within the chorion layers as in other Macrobiotidae species (Fig. 13C–F and 14C–D). The microgranulation is present on the egg chorion surface under the processes, however it is visible only in SEM and only when processes are broken or detached from the chorion surface (Fig. 14C and D).

3.3.4.3. Reproduction. Neither testis in males nor spermathecae filled with sperm in gravid females have been found on the freshly prepared slides. Also, specimens of the Tanzanian population did not exhibit sexual dimorphism such as lateral gibbosities on legs IV. Thus, most likely the species is parthenogenetic.

3.3.4.4. Genetic comparisons. The 18S rRNA and ITS-2 exhibited single haplotypes, but in 28S rRNA and COI, two haplotypes were found (Table 2), with p-distances of 0.1% and 2.1%, respectively. The ranges of uncorrected genetic p-distances between the Tanzanian
M. pentannulatus and other Minibiotus species, for which sequences are available from GenBank, are as follows:

**18S rRNA**: 2.0–4.0% (3.3% on average), with the most similar being *M. ioculator* sp. nov. from the Republic of South Africa (MT023998) and the least similar being unidentified species of the *M. intermedius* group from Italy (HQ604980);

**28S rRNA**: comparison with only two species: 7.2 and 7.3% difference with *M. ioculator* sp. nov. from the Republic of South Africa (MT024041) and 12.2 and 12.3% difference with *M. gumersindoi* from Spain (FJ435761);

**ITS-2**: comparison with only one species: 19.2% difference with *M. ioculator* sp. nov. from the Republic of South Africa (MT024000);

**COI**: comparison with only two species: 21.1% difference with *M. ioculator* sp. nov. from the Republic of South Africa (MT023412) and 23.9–24.4% difference with *M. gumersindoi* from Spain (FJ435803).

### 4. Discussion

Minibiotus was established 40 years ago by Schuster et al. (1980) who extracted several species from the genus *Macrobiotus* that were characterized by uniform morphology. Initially, however, the erection of *Minibiotus* was not commonly accepted and it was met with criticism by Pilato (1982) and Ramazzotti & Maucci (1983) who treated this genus invalid because they considered its diagnosis vague. Nevertheless, with time, the genus was eventually accepted and it currently comprises nearly fifty species. The first and so far the only revision of the genus by Claxton (1998) included descriptions of numerous new species, aiding species identification and further descriptions. Although the taxonomic status of *Minibiotus* is no longer challenged, the extreme morphological diversity within the genus presented by Claxton (1998) and later species descriptions clearly indicate that *Minibiotus* is likely to hold at least several distinct evolutionary lineages that could potentially be described as separate genera (Stec et al. 2015). Specifically, *Minibiotus* comprises species with two and three macroplacoids in the pharynx or species without and with pores and the latter can be further divided into species with solely circular pores and species with a mixture of circular and star-shaped pores. Recent phylogenetic works show that morphological traits such as the number of macroplacoids and even their shape and spatial arrangement or the presence of cuticular pores are stable at the genus level (e.g. see the following genera that were established with integrative data in which the macroplacoid number and morphology were used as major diagnostic characters: *Paramacrobiotus*, *Mesobiotus* or *Pilatobius*). Apart from *Minibiotus*, there are also other tardigrade genera that were erected before the molecular phylogenetics era, solely with morphological data and that comprise species with varying numbers of macroplacoids (e.g. *Doryphoribius* and *Adropion*). Importantly, however, their phylogenies have not been thoroughly studied, thus it is likely that these genera are not monophyletic either (see Gasiorek et al. 2019). Moreover, some of the *Minibiotus* species originally described with two macroplacoids have recently been transferred to the *Macrobiotus hufelandi* group after a careful PCM analysis (i.e. *M. acadianus* and *M. julianae* in Stec et al., 2015). Similarly, in all tardigrade genera that were verified by methods of molecular phylogenetics, cuticle is either porous or poreless, suggesting that this trait is typically conserved at a genus
level (e.g. Paramacrobiotus and Mesobiotus; Guidetti et al., 2009; Vecchi et al., 2016; Kaczmirek et al., 2018; Guidetti et al., 2019; Guil et al., 2019; Stec et al., 2020). Importantly, the morphological hypothesis that Minibiotus is not monophyletic is also supported by the limited available genetic data (Guil & Giribet 2012; Bertolani et al. 2014). For example, Bertolani et al. (2014) showed that Minibiotus furcatus is not directly related to the few other sequenced Minibiotus species, but it clusters with the genus Paramacrobiotus. Moreover, the species exhibits spermatozoa similar to those observed in some Paramacrobiotus spp. and its mouth is surrounded by a ring of ten short peribuccal lamellae (instead of papulae that are supposed to be present in Minibiotus; but see the paragraph below). Thus, Bertolani et al. (2014) provisionally moved M. furcatus back to a ‘basket genus’ Macrobiotus. However, the miniaturised lamellae in Macrobiotus furcatus have a clearly different morphology compared to lamellae in Macrobiotus, Paramacrobiotus or Mesobiotus. Thus, taking into consideration both molecular phylogeny and morphology, it is likely that M. furcatus represents a separate genus that is yet to be described when more data are available.

The original general diagnosis of the genus has been refined in later studies (Binda & Pilato 1992; Claxton 1998; Guil & Guidetti 2005; Guidetti et al. 2007; Stec et al. 2015) and currently a species is considered as representing Minibiotus if its buccal apparatus conforms to the list of ten characters (see Stec et al. 2015 for the latest version of the list). The two species analysed in this study meet all these ten criteria and thus can be considered as Minibiotus species according to current standards. However, our study signals a potential problem with one of the key traits defining the genus, namely the peribuccal papulae. These structures are hardly visible under LCM, and SEM data for Minibiotus species are still extremely scarce and of varying quality (only three works comprise such SEM photographs: Schuster et al. 1980; Claxton 1998; the present study). As a result, the morphology of
peribuccal papulae in *Minibiotus* has never been described in detail. With currently existing data, it is hard to determine whether peribuccal structures in *Minibiotus* should be considered as true papule (as it has been shown in Fig. 5 by Schuster et al. 1980) or rather as shortened and thickened lamellae packed closely to each other (as it has been shown in Fig. 1 by Claxton 1998 and in this study in Fig. 4C and D and 12C–D). Our photographs, being of the highest resolution among the published images, indicate that the latter scenario is more likely. However, much more effort should be made towards increasing the species sample size regarding SEM documentation of peribuccal structures in various *Minibiotus* to allow a formulation of a confident conclusion on their morphology.

Our conclusions are in line with previous studies on *Minibiotus* (Guidetti et al., 2007; Bertolani et al., 2014; Stec et al., 2015) and indicate that the genus should be considered polyphyletic. It is also obvious that this tardigrade group requires a thorough revision with an integrative taxonomy approach to solve the highlighted problem with the genus diagnosis as well as to test the hypothesis on putative new genera potentially hidden within the genus *Minibiotus*.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Fig. 14.** *M. pentannulatus* Londono et al., 2017 from Tanzania – egg chorion seen in SEM. (A) Entire egg. (B–C) Details of the egg surface. (D–F) Details of the processes. Empty flat arrowheads indicate undevolved annulations, filled flat arrowheads indicate microgranulation under egg processes whereas filled indented arrowheads indicate internal thickenings (attachaments/sutures) at the processes bases. Scale bars in μm.
Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcz.2020.03.007.

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