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An Integrative Description of Two New *Mesobiotus* Species (Tardigrada: Eutardigrada: Macrobiotidae) with Updated Genus Phylogeny

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This work presents two new *Mesobiotus* species from the Republic of South Africa, formally described using integrative analyses. Specimens of the new species are examined in terms of morphology and morphometry under a contrast phase light microscope (PCM) and scanning electron microscope (SEM). For both new species, genetic data in the form of DNA sequences of commonly used molecular markers are also provided (18S rRNA, 28S rRNA, *COI*, ITS-2). Furthermore, such genetic data are also provided for the first time for *Mesobiotus peterseni* (Maucci, 1991) from Greenland. The study also presents a multilocus molecular phylogeny of the genus and an elaborated discussion on the taxa groupings and species composition. This results in the ratification of three informal morpho-groups in order to ease and improve communication in further taxonomic studies on the genus. Finally, an updated key to all valid nominal *Mesobiotus* taxa (71 species) is provided to enhance species identification in this morphologically diverse group of limno-terrestrial tardigrades.

Key words: Egg ornamentation, Integrative taxonomy, *Mesobiotus diegoi* sp. nov., *Mesobiotus maklowiczi* sp. nov., Tardigrades.

BACKGROUND

The phylum Tardigrada is a microinvertebrate group that comprises more than 1400 species (Guidetti and Bertolani 2005; Degma and Guidetti 2007 2022). Tardigrades are water-dependent animals that require at least a film of water surrounding their body to perform all life functions. However, many tardigrade taxa are known for their ability to enter cryptobiosis, a diapause stage in which they resist adverse environmental conditions such as desiccation and freezing (*e.g.*, Guidetti et al. 2011 2012; Wełnicz et al. 2011; Kaczmarek et al. 2019). As a result, tardigrades can be considered a cosmopolitan group of animals that inhabit terrestrial, freshwater, and marine environments throughout the world (Nelson et al. 2019).

One of the most speciose limno-terrestrial and softbodied tardigrade groups is the family Macrobiotidae, within which 14 distinct genera are currently recognized (Stec et al. 2021; Degma and Guidetti 2022). Importantly, the contribution of these genera to the total number of species in the family is disproportionately distributed, with four genera contributing most of the taxa, namely Macrobiotus C.A.S. Schultze, 1834, Mesobiotus Vecchi, Cesari, Bertolani, Jönsson, Rebecchi and Guidetti, 2016, Minibiotus Schuster, 1980 in Schuster et al. (1980) and Paramacrobiotus Guidetti, Schill, Bertolani, Dandekar and Wolf, 2009. For many years, most of these macrobiotid genera (including the last three mentioned above as flag examples) had been recognized as informal groups or complexes within the genus Macrobiotus that were later elevated to the genus

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level (Schultze 1834; Schuster et al. 1980; Vecchi et al. 2016; Guidetti et al. 2009; Stec et al. 2021). According to recent comprehensive phylogenetic studies focusing partially or wholly on the family Macrobiotidae, most genera turned out to be monophyletic except *Minibiotus*, which was always recovered as paraphyletic (Bertolani et al. 2014; Guil et al. 2019; Stec et al. 2021).

The present study focuses on the genus *Mesobiotus* which currently comprises 73 nominal species, out of which four are designated as nomina inquirenda (Kaczmarek et al. 2020; Degma and Guidetti 2022). The nomenclatural birth of the genus starts with its erection by Vecchi et al. (2016). The erection was supported by morphological and genetic data which congruently showed that two formerly recognized informal morphogroups, the Macrobiotus harmsworthi group, and the Macrobiotus furciger group, form a monophyletic clade. The first study that conducted a more detailed investigation into the relationship within the genus was Kaczmarek et al. (2018) who also redescribed Mesobiotus harmsworthi (Murray, 1907a) which constitutes the type species for the genus. This study was followed by several subsequent investigations that also looked at genealogical relations between Mesobiotus taxa (Kaczmarek et al. 2020; Stec 2021; Stec et al. 2021 2022; Short et al. 2022). Family-level phylogenetic investigations in these studies confirmed the monophyly of the genus, while all of them also reported a lack of congruence between morphology and genetics within this group. Namely, both traditionally recognized morpho-groups (harmsworthi and furciger groups) did not form monophyletic clades, but intermixed in all genus phylogenies published so far.

Here, by means of integrative taxonomy, I describe two new Mesobiotus species from the Republic of South Africa. Both descriptions are based on detailed morphological and morphometric investigations with light-contrast-phase (PCM) and scanning electron microscopes (SEM). Furthermore, the phenotypic data presented in each description are associated with genetic data in the form of DNA sequences of molecular markers commonly used in tardigrade taxonomy (18S rRNA, 28S rRNA, ITS-2, COI). Additionally, for the first time, genetic data for Mesobiotus peterseni (Maucci, 1991) based on specimens from a newly discovered population in Greenland are also reported. Finally, I also present an upgraded multilocus molecular phylogeny of the genus and discuss the taxa compositions within Mesobiotus morpho-groups.

MATERIALS AND METHODS

Sample processing

Two lichen samples containing new species were collected in the Republic of South Africa. Specifically, sample ZA.001 was collected in Giants Castle Game Reserve, KwaZulu-Natal whereas sample ZA.002 in Groot Swartberg Nature Reserve, Western Cape. The samples were collected by Witold Morek and Bartłomiej Surmacz in September 2018 from rocks. The samples were examined for terrestrial tardigrades using standard methods as described in Stec et al. (2015). In order to perform integrative taxonomic descriptions, the isolated animals and eggs extracted from both samples were split into three groups for specific analyses: morphological analysis with phase contrast light microscopy, morphological analysis with scanning electron microscopy, and DNA sequencing (for details please see sections "Material examined" provided below for each description). Additionally, a mixed sample of moss and lichen collected in arctic tundra in Greenland was examined (60°28'1.5"N, 45°34'27.8"W; 24.08.2014, leg. Lars Engberg Hansen). The sample contained animals and eggs of M. peterseni, and the eggs were used to obtain DNA sequences of that species.

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 then dried for five to seven days at 60°C. Dried slides were sealed with a transparent nail polish and examined under a Leica DMLB light microscope with phase contrast (PCM), associated with a digital camera. Immediately after mounting the specimens in the medium, slides were checked under PCM for the presence of males and females in the studied population, as the spermatozoa in testis and vas deferens are visible only for several hours after mounting (Coughlan et al. 2019; Coughlan and Stec 2019). In order to obtain clean eggs for SEM, eggs were processed according to the protocol by Stec et al. (2015). In short, eggs were first subjected to a water/ethanol and an ethanol/acetone series, then to CO₂ critical point drying and finally sputter coated with a thin layer of gold. Specimens were examined under high vacuum in a Versa 3D DualBeam Scanning Electron Microscope at the ATOMIN facility of the Jagiellonian University, Kraków, Poland. All figures were assembled in Corel Photo-Paint X6. For structures that could not be satisfactorily focused in a single photograph, a stack of 2-6 images were taken with an equidistance of ca.

 $0.2\ \mu m$ and assembled manually into a single deep-focus image.

Morphometrics and morphological nomenclature

All measurements are given in micrometres (µm). Sample size was adjusted following recommendations by Stec et al. (2016a). 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 buccal apparatus and claws were classified according to Pilato and Binda (2010) and Vecchi et al. (2016), respectively. The terminology used to describe oral cavity armature and egg shell morphology follows Michalczyk and Kaczmarek (2003). Macroplacoid length sequence is given according to Kaczmarek et al. (2014) whereas morphological states of cuticular bars on legs follow Kiosya et al. (2021). 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 and Michalczyk (2017). Morphometric data were handled using the "Parachela" ver. 1.8 template available from the Tardigrada Register (Michalczyk and Kaczmarek 2013) and are given in Supplementary Materials (SM. 1 and 2). Tardigrade taxonomy follows Bertolani et al. (2014) and Stec et al. (2021).

DNA sequencing

The DNA was extracted from individual animals following a *Chelex*[®] 100 resin (*Bio-Rad*) extraction method by Casquet et al. (2012) with modifications described in detail in Stec et al. (2020). Before extraction all animals were checked *in-vivo* under microscope. Four DNA fragments differing in mutation

rates were sequenced. Namely: the small ribosomal subunit (18S rRNA, nDNA), the large ribosomal subunit (28S rRNA, nDNA), the internal transcribed spacer (ITS-2, nDNA), and the cytochrome oxidase subunit I (*COI*, mtDNA). All fragments were amplified and sequenced according to the protocols described in Stec et al. (2020); primers are listed in table 1. Sequencing products were read with the *ABI 3130x1* sequencer at the Genomed company (Warsaw, Poland). Sequences were processed in *BioEdit* ver. 7.2.5 (Hall 1999) and submitted to GenBank. Prior submission all obtained *COI* sequences were translated into protein sequences in *MEGA11* (Tamura et al. 2021) to check against pseudogenes.

Phylogenetic analysis

To establish phyletic positions of both new species and M. peterseni a phylogenetic tree was constructed. For this purpose a data set was compiled from taxa/ specimens for which DNA sequences of at least two (out of all four analysed in this study) molecular markers are available and suitable for concatenation (Table 2). Sequences of four analysed DNA fragments of Macrobiotus kamilae Coughlan and Stec, 2019 and Macrobiotus hannae Nowak and Stec, 2018 were used as the outgroup. The sequences were aligned using the AUTO method (for COI and ITS-2) and the Q-INS-I method (for ribosomal markers: 18S rRNA and 28S rRNA) of MAFFT version 7 (Katoh et al. 2002; Katoh and Toh 2008) and manually checked against non-conservative alignments in BioEdit. Then, the aligned sequences were trimmed to: 1010 (18S rRNA), 774 (28S rRNA), 559 (ITS-2), 658 (COI) bp and concatenated using SequenceMatrix (Vaidya et al. 2011). Before partitioning, the concatenated alignment was divided into 6 data blocks constituting three separate blocks of ribosomal markers and three separate blocks of three codon positions in the COI data set. Using PartitionFinder (Lanfear et al. 2016) under the

 Table 1. Primers with their original references used for amplification of the four DNA fragments sequenced in the study

DNA marker	Primer name	Primer direction	Primer sequence (5'-3')	Primer source
18S rRNA	18S_Tar_Ff1	forward	AGGCGAAACCGCGAATGGCTC	Stec et al. (2017)
	18S_Tar_Rr1	reverse	GCCGCAGGCTCCACTCCTGG	
28S rRNA	28SF0002	forward	GRCRAGAKTACCCGCTGAAC	This study
	28SR0990	reverse	CCTTGGTCCGTGTTTCAAGAC	Mironov et al. (2012)
ITS-2	ITS2_Eutar_Ff	forward	CGTAACGTGAATTGCAGGAC	Stec et al. (2018a)
	ITS2_Eutar_Rr	reverse	TCCTCCGCTTATTGATATGC	
COI	LCO1490-JJ	forward	CHACWAAYCATAAAGATATYGG	Astrin and Stüben (2008)
	HCO2198-JJ	reverse	AWACTTCVGGRTGVCCAAARAATCA	

Akaike Information Criterion (AIC), the best scheme of partitioning and substitution models were chosen for Bayesian phylogenetic analysis. Bayesian inference (BI) marginal posterior probabilities were calculated for the concatenated (18S rRNA + 28S rRNA + ITS-2 + COI) data set using MrBayes v3.2 (Ronquist and Huelsenbeck 2003). Random starting trees were used and the analysis was run for ten million generations,

Table 2. Sequences used for phylogenetic analysis. Bold font indicate sequences obtained in this study

Species	18S rRNA	28S rRNA	ITS-2	COI	Source
Mesobiotus diegoi sp. nov.	OP142527	OP142520	OP142514	OP143858	this study
	OP142526	OP142521	OP142515	OP143857	this study
Mesobiotus maklowiczi sp. nov.	OP142525	OP142518		OP143855	this study
	OP142524	OP142519		OP143856	this study
Mesobiotus peterseni (Maucci, 1991)	OP142528	OP142522	OP142516	OP143859	this study
	OP142529	OP142523	OP142517	OP143860	this study
M. ethiopicus Stec and Kristensen, 2017	MF678793	MF678792	MN122776	MF678794	Stec and Kristensen (2017), Stec (2019)
M. datanlanicus Stec, 2019	MK584659	MK584658	MK584657	MK578905	Stec (2019)
M. dilimanensis Itang et al., 2020	MN257048	MN257049	MN257050	MN257047	Itang et al. (2020)
M. philippinicus Mapalo et al., 2016	KX129793	KX129794	KX129795	KX129796	Mapalo et al. (2016)
M. insanis Mapalo et al., 2017	MF441488	MF441489	MF441490	MF441491	Mapalo et al. (2017)
M. hilariae Vecchi et al., 2016	KT226070			KT226108	Vecchi et al. (2016)
M. radiatus (Pilato et al., 1991)	MH197153	MH197152	MH197267	MH195147	Stec et al. (2018b)
			MH197268	MH195148	Stec et al. (2018b)
M. romani Roszkowska et al., 2018	MH197158	MH197151	MH197150	MH195149	Roszkowska et al. (2018)
M. harmsworthi (Murray, 1907a)	MH197146	MH197264	MH197154	MH195150	Kaczmarek et al. (2018)
				MH195151	Kaczmarek et al. (2018)
M. occultatus Kaczmarek et al., 2018	MH197147		MH197155	MH195152	Kaczmarek et al. (2018)
M. furciger group species NO	MH197148	MH197265	MH197156	MH195153	Kaczmarek et al. (2018)
M. harmsworthi group species RU	MH197149	MH197266	MH197157	MH195154	Kaczmarek et al. (2018)
M. fiedleri Kaczmarek et al., 2020	MH681585	MH681693	MH681724	MH676056	Kaczmarek et al. (2020)
M. anastasiae Tumanov, 2020	MT903468	MT903612	MT903470	MT904513	Tumanov (2020)
M. skoracki Kaczmarek et al., 2018		MW680636		MW656257	Kayastha et al. (2021)
M. imperialis Stec, 2021	OL257854	OL257866		OL311514	Stec (2021)
1	OL257855	OL257867		OL311515	Stec (2021)
M. marmoreus Stec, 2021	OL257856	OL257868	OL257861	OL311516	Stec (2021)
,	OL257857	OL257869	OL257862	OL311517	Stec (2021)
	OL257858	OL257870	OL257863	OL311518	Stec (2021)
Mesobiotus cf. barabanovi	MN310392	MN310388	MN310390	MN313170	Kaczmarek et al. (2020)
Mesobiotus sp. Macro07 042	MW751942			MW727957	Short et al. (2022)
Mesobiotus cf. furciger Macro06 296	MW751936			MW727958	Short et al. (2022)
Mesobiotus cf. furciger Macro06 310	MW751937			MW727961	Short et al. (2022)
Mesobiotus cf. furciger Macro06 313	MW751939			MW727960	Short et al. (2022)
Mesobiotus cf. furciger CC MF 4	MW751949			MW727933	Short et al. (2022)
Mesobiotus cf. furciger ABDC MF 3	MW751944			MW727932	Short et al. (2022)
Mesobiotus cf. furciger KPRI MF 1	MW751962			MW727934	Short et al. (2022)
Mesobiotus cf. furciger HMI MF 1	MW751957			MW727941	Short et al. (2022)
Mesobiotus cf. furciger EBNI MF 2	MW751952			MW727937	Short et al. (2022)
Mesobiotus cf. furciger EBNI MF 4	MW751954			MW727938	Short et al. (2022)
Mesobiotus cf. furciger PSAL MF 2	MW751967			MW727939	Short et al. (2022)
Mesobiotus cf. furciger Macro06 162	MW751934			MW727955	Short et al. (2022)
Mesobiotus cf. furciger Macro06 171	MW751935			MW727956	Short et al. (2022)
Mesobiotus cf. furciger IN07 MF 1	MW751959			MW727951	Short et al. (2022)
Mesobiotus cf. furciger IN07 MF 4	MW751960			MW727953	Short et al. (2022)
Mesobiotus of furciger IN07 MF 8	MW751961			MW727947	Short et al. (2022)
Mesobiotus cf. furciger FN01 MF 6	MW751955			MW727945	Short et al. (2022)
Macrobiotus kamilae Coughlan and Stee 2019	MK737070	MK737064	MK737067	MK737920	Coughlan and Stec (2019)
				MK737921	Coughlan and Stee (2019)
Macrobiotus hannae Nowak and Stee 2018	MH063922	MH063924	MH063923	MH057764	Nowak and Stee (2018)

sampling the Markov chain every 1000 generations. An average standard deviation of split frequencies of < 0.01was used as a guide to ensure the two independent analyses had converged. The program Tracer v1.6 (Rambaut et al. 2014) was then used to ensure Markov chains had reached stationarity and to determine the correct 'burn-in' for the analysis, which was the first 10% of generations. The ESS values were greater than 200 and the consensus tree was obtained after summarising the resulting topologies and discarding the 'burn-in'. ModelFinder (Kalyaanamoorthy et al. 2017) was used to choose the best-fit models according to the AIC for Maximum Likelihood (ML) analysis. Then, ML reconstruction was conducted using W-IQ-TREE (Nguyen et al. 2015; Trifinopoulos et al. 2016). One thousand ultrafast bootstrap (UFBoot) replicates were applied to provide support values for branches (Hoang et al. 2018). The consensus tree was viewed and visualised by FigTree v.1.4.3 available at http://tree.bio. ed.ac.uk/software/figtree. The best evolutionary models of sequence evolution selected for BI and ML analyses are given in supplementary materials (SM. 3).

RESULTS

TAXONOMY

Phylum: Tardigrada Doyère, 1840 Class: Eutardigrada Richters, 1926 Order: Parachela Schuster, Nelson, Grigarick and Christenberry, 1980 Superfamily: Macrobiotoidea Thulin, 1928 (in Marley et al. 2011) Family: Macrobiotidae Thulin, 1928 Genus: *Mesobiotus* Vecchi, Cesari, Bertolani, Jönsson, Rebecchi and Guidetti, 2016

Mesobiotus diegoi sp. nov. (Figs. 1–6; Tables 3–4) urn:lsid:zoobank.org:act:2C2D67EE-A0CC-46EC-A5E0-30B71554A8C8

Material examined: 68 animals, 28 eggs mounted on microscope slides in Hoyer's medium (some of the eggs were embryonated), eight eggs examined in SEM and two specimens processed for DNA sequencing.

Type locality: 29°16'5.1"S, 29°30'48.6"E; 1756 m asl: Giants Castle Game Reserve, Drakensberg National Park, KwaZulu-Natal, Republic of South Africa, lichen growing on rock in mountainous grassland, coll. Witold Morek and Bartłomiej Surmacz, 16 September 2018.

Etymology: The species is named after my good friend Diego Fontaneto, a world-known rotiferologist

and meiofauna specialist working in the Water Research Institute of the National Research Council (Verbania, Italy).

Type depositories: Holotype (\mathcal{P}): slide ZA.001.06 with 2 paratypes and 62 paratypes (slides: ZA.001.*, where the asterisk can be substituted by any of the following numbers: 01–04, 07–08) and 20 eggs (slides: ZA.001.*: 09–11) are deposited at the Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Sławkowska 17, 31-016, Kraków, Poland, whereas 3 paratypes (slide: ZA.001.05) and 6 eggs (slide: ZA.001.12) are deposited at the Department of Animal Taxonomy and Ecology, Adam Mickiewicz University in Poznań, Umultowska 89, Poznań, Poland.

Animals (measurements and statistics in Table 3): Body almost transparent in small specimens and whitish in adults; after fixation in Hoyer's medium body transparent (Fig. 1A). Eyes present in alive animals and dissolved by Hoyer's medium in approximately 65% of all mounted specimens. Body cuticle smooth, *i.e.*, without pores, body granulation, sculpturing, or tubercles. A fine granulation is present on the external surface of legs I–III (Fig. 1B) that extends through the frontal leg surface to the internal surface where it is present mainly in the cuticular fold (Fig. 1C). Granulation is also present on the lateral and dorsal surfaces of legs IV (Fig. 1D). A cuticular bulge, similar to a pulvinus, is present on the internal surface of legs I-III (Fig. 1C). Claws of the Mesobiotus type, with a peduncle connecting the claw to the lunula, a basal septum, and well-developed accessory points situated parallel to the primary branch (Fig. 2A-B). Lunulae under claws I-III smooth (Fig. 2A) and those under claws IV slightly dentate (Fig. 2B-C). A single continuous cuticular bar with shadowed extensions narrowing toward double muscle attachments is present below claws I-III (Figs. 1C, 2A), while a horseshoeshaped structure connects the anterior and posterior lunulae on claws IV (Fig. 2B).

Mouth antero-ventral. Bucco-pharyngeal apparatus of the *Macrobiotus* type (Fig. 3A), with ventral lamina and ten small peribuccal lamellae. The oral cavity armature well developed and consists of three bands of teeth (Fig. 3B–C). The first band of teeth is composed of numerous small granules arranged in several discrete rows located anteriorly in the oral cavity, just behind the bases of the peribuccal lamellae (Fig. 3B–C). The second band of teeth is located between the ring fold and the third band of teeth and is composed of ridges parallel to the main axis of the buccal tube that are larger than those in the first band (Fig. 3B–C). The teeth of the third band are located within the posterior portion of the oral cavity, between the second band of teeth and the opening of the buccal tube (Fig. 3B–C). The third band of teeth is discontinuous and divided into a dorsal and ventral portion. Under PCM, dorsal teeth are visible as two lateral and one median transverse ridges/ crests (Fig. 3B) whereas ventral teeth consist of two lateral transverse ridges/crests between which usually one round or trapezoidal ventro-median tooth is present (Fig. 3C). Sometimes, additional granular teeth are present between the second and third band of teeth (Fig. 3C). Pharyngeal bulb ovoid (Fig. 3A), with triangular apophyses, three rod-shaped macroplacoids, and a large, elongated drop-shaped microplacoid placed close to the third macroplacoid (Fig. 3D–E). The macroplacoid length sequence is 2 < 3 < 1. The first macroplacoid is

anteriorly narrowed, and the third has a clearly defined subterminal constriction (Fig. 3D–E).

Eggs (measurements and statistics in Table 4): White, laid free, spherical in shape and equipped with large and long conical processes (Figs. 4A–F, 5A–F). Egg surface between the processes without areolation. In PCM the egg surface between processes seems to be rough with dark bars/wrinkles and faintly light refracting dots (Fig. 4A–B) whereas in SEM the surface is clearly wrinkled with bulging wrinkles radiating out from the process bases (Fig. 5E–F). Small pores (up to 0.3 μ m) are scattered across the interprocess surface and are mainly distributed in the depression between

Table 3. Measurements [in μ m] and *pt* values of selected morphological structures of animals of *Mesobiotus diegoi* sp. nov.; specimens mounted in Hoyer's medium

Character	N Range		nge	e Mean		S	D	Holotype	
		μm	pt	μm	pt	μm	pt	μm	pt
Body length	20	334–712		534		108		712	
Buccal tube									
Buccal tube length	20	35.1-63.5	_	51.4	_	9.7	_	61.4	_
Stylet support insertion point	20	26.4-49.1	75.1–77.5	39.3	76.4	7.6	0.7	46.9	76.4
Buccal tube external width	20	5.3-10.9	15.1–17.7	8.6	16.7	1.8	0.7	10.1	16.4
Buccal tube internal width	20	4.0-8.5	11.4–13.5	6.6	12.8	1.5	0.6	8.1	13.2
Ventral lamina length	18	20.9-41.3	57.9-66.2	32.4	62.5	6.2	2.3	37.1	60.4
Placoid lengths									
Macroplacoid 1	20	5.3-12.8	14.7–21.4	9.8	18.7	2.6	1.9	12.7	20.7
Macroplacoid 2	20	3.5-8.6	10.0–14.4	6.3	12.1	1.6	1.2	6.8	11.1
Macroplacoid 3	20	4.7-11.4	12.6–18.5	8.3	15.9	2.3	1.8	8.7	14.2
Microplacoid	20	3.3-9.9	8.2–16.1	5.5	10.6	1.7	1.7	9.9	16.1
Macroplacoid row	20	16.6-36.5	43.9–57.9	27.3	52.4	6.8	4.1	32.7	53.3
Placoid row	20	21.2-43.0	54.3-71.3	33.3	64.3	7.9	4.2	42.2	68.7
Claw I heights									
External primary branch	20	9.4-16.3	24.1–28.8	13.2	25.9	2.1	1.3	14.8	24.1
External secondary branch	12	7.3-13.8	19.8–23.9	11.2	21.3	2.1	1.2	13.8	22.5
Internal primary branch	20	7.8-15.0	21.3–28.2	12.5	24.4	2.1	1.6	14.2	23.1
Internal secondary branch	16	7.2-12.8	18.0-22.9	10.6	20.2	1.9	1.4	12.8	20.8
Claw II heights									
External primary branch	20	10.0-17.1	25.7-28.9	13.9	27.1	2.3	1.1	16.3	26.5
External secondary branch	18	7.5-14.6	19.5–24.5	11.7	22.0	1.9	1.3	14.2	23.1
Internal primary branch	20	8.4-16.1	23.2-27.7	13.0	25.3	2.3	1.3	15.4	25.1
Internal secondary branch	18	7.4-13.5	19.0-22.4	10.6	20.9	2.0	1.1	12.9	21.0
Claw III heights									
External primary branch	19	9.7-17.7	25.4-29.6	14.0	27.4	2.4	1.3	17.7	28.8
External secondary branch	15	7.4-13.5	19.5-25.4	11.5	22.0	1.8	1.5	13.5	22.0
Internal primary branch	18	8.8-16.4	23.0-27.7	13.2	25.6	2.3	1.5	14.9	24.3
Internal secondary branch	16	7.4-13.6	18.3–24.9	10.8	21.2	2.0	1.5	13.6	22.1
Claw IV heights									
Anterior primary branch	20	9.8-21.7	27.3–35.3	15.8	30.8	3.1	2.5	21.7	35.3
Anterior secondary branch	18	8.2-16.1	20.3-27.2	12.5	24.3	2.2	1.8	16.1	26.2
Posterior primary branch	20	11.5-22.1	29.6–36.0	16.8	32.8	3.0	1.7	22.1	36.0
Posterior secondary branch	18	8.6–16.8	20.7–28.6	13.1	25.7	2.6	1.8	16.8	27.4

N, number of specimens/structures measured. Range, refers to the smallest and the largest structure among all measured specimens. SD, standard deviation.

the bulged wrinkles. The pores are clearly visible in SEM (Fig. 5A–E), but under PCM they are seen as the mentioned faintly light-refracting dots (Fig. 4). The bases of egg processes are surrounded by a crown

of strong thickenings that are evident only in PCM (Fig. 4A–B). The egg processes are evenly spaced, having flexible upper portion often elongated into short filament (only sometimes bifurcation or trifurcation is



Fig. 1. *Mesobiotus diegoi* sp. nov. – PCM image of habitus and leg's cuticle morphology: (A) dorso-ventral projection (holotype); (B) granulation on the external surface of leg II (paratype); (C) granulation and a pulvinus-like cuticular bulge on the internal surface of leg III (holotype); (D) granulation on the dorsal and lateral surface of leg IV (holotype). Filled flat arrowheads indicate cuticular fold and granulation on the internal leg surface. Scale bar in µm.



Fig. 2. Mesobiotus diegoi sp. nov. – PCM images of claws: (A) claws II with smooth lunulae (paratype); (B) claws IV (paratype); (C) lunula IV with dentate margin (paratype). Filled flat arrowhead indicates a single continuous cuticular bar below the claws, empty flat arrowheads indicate paired muscle attachments, and filled indented arrowhead indicates a horseshoe structure connecting the anterior and the posterior claw. Scale bars in µm.

present; Figs. 4C–F, 5A–C). Often, within the upper portion of the egg processes, below the flexible part a bubble-like structure is present and visible in the midsection of the process (Fig. 4C–F). In SEM only the surface of this upper part of the egg process (about 50% of the entire process length) is punctured with micropores (0.15–0.20 μ m in size). The labyrinthine layer is visible under PCM as a reticulum in the process walls, with varying mesh size uniformly distributed within the process walls, except for the ring of basal meshes that are clearly larger than the meshes above them (Fig. 4A–B). In SEM, the process walls are evenly



Fig. 3. *Mesobiotus diegoi* sp. nov. – PCM images of the buccal apparatus: (A) an entire buccal apparatus (paratype); (B–C) the oral cavity armature, dorsal and ventral teeth respectively (paratype); (D–E) placoid morphology, dorsal and ventral placoids, respectively (paratype). Filled flat arrowheads indicate the first band of teeth, empty flat arrowheads indicate the second band of teeth, filled indented arrowheads indicate the third band of teeth, and empty indented arrowheads indicate subterminal constrictions in the third macroplacoid. Scale bars in µm.

annulated at their entire length (Fig. 5A–F). The flexible upper portions of the egg processes are smooth and not covered with granules (Fig. 5C).

Reproduction: The new species is dioecious. Spermathecae filled with sperm have not been found in

gravid females on freshly prepared slides. However, in males, the testes, filled with sperm, are clearly visible under PCM up to 48 hours after mounting in Hoyer medium (Fig. 6). The new species does not exhibit male secondary sexual dimorphism traits such as lateral



Fig. 4. *Mesobiotus diegoi* sp. nov. – PCM images of the egg. (A–B) egg surface; (C–F) egg processes midsections. Filled flat arrowheads indicate a ring of large basal meshes in the egg process reticulum (labirynthine layer), empty flat arrowheads indicate crowns of thickenings around the processes bases. Scale bars in μ m.

Table 4.	Measuremen	nts [in µm]	of the eggs	of Mesobiotus	diegoi sp.	. nov.; eggs	mounted in	h Hoyer's	medium; p	process
base/heig	ght ratio is exp	pressed as j	percentage							

Character	Ν	Range	Mean	SD
Egg bare diameter	18	71.9–91.0	82.6	4.4
Egg full diameter	18	141.0-178.9	160.3	10.8
Process height	54	30.7-47.3	39.8	3.7
Process base width	54	17.8-27.3	22.4	1.8
Process base/height ratio	54	45%-68%	57%	5%
Inter-process distance	54	2.3-4.5	3.5	0.6
Number of processes on the egg circumference	18	10–12	11.2	0.6

N, number of eggs/structures measured. Range, refers to the smallest and the largest structure among all measured specimens. SD, standard deviation.



Fig. 5. *Mesobiotus diegoi* sp. nov. – SEM images of eggs: (A–B) entire view of the egg; (C–D) egg processes; (E–F) details of the egg surface between processes. Scale bars in µm.

gibbosities on legs IV.

DNA sequences:

The sequences obtained for all four molecular markers analysed in this study were of good quality and were represented by single haplotypes.

The 18S rRNA sequences (GenBank: OP142526, OP142527), 1020 bp long;

The 28S rRNA sequences (GenBank: OP142520, OP142521), 712 bp long;

The ITS-2 sequences (GenBank: OP142514, OP142515), 354 bp long;

The *COI* sequences (GenBank: OP143857, OP143858), 658 bp long.

Mesobiotus maklowiczi sp. nov.

(Figs. 7–12; Tables 5–6) urn:lsid:zoobank.org:act:15A610D7-7997-4491-92EA-AE9F8D6CAB9A

Material examined: 29 animals, 51 eggs mounted on microscope slides in Hoyer's medium (some of the eggs were embryonated), 10 eggs examined in SEM and two specimens processed for DNA sequencing.

Type locality: 33°20'32"S, 21°53'31"E; 1004 m asl: Groot Swartberg Nature Reserve, Western Cape, Republic of South Africa, lichen growing on rock, coll. Witold Morek and Bartłomiej Surmacz, 6 September 2018.

Etymology: The species is named after Robert Makłowicz, who is a journalist, historian, and culinary expert that beautifully promotes European cuisine and slow food. He lives in Kraków and runs his own

YouTube channel that I enjoy watching. He is also a hat lover, and the egg processes of the new species resemble a funny peaked hat that Robert would be surely eager to try on.

Type depositories: Holotype (\mathcal{P}): slide ZA.002.01 with 1 paratype and 25 paratypes (slides: ZA.002.*, where the asterisk can be substituted by any of the following numbers: 02–05) and 46 eggs (slides: ZA.002.*: 07–13) are deposited at the Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Sławkowska 17, 31–016, Kraków, Poland whereas 2 paratypes (slide: ZA.002.06) and 5 eggs (slide: ZA.002.14) are deposited at the Department of Animal Taxonomy and Ecology, Adam Mickiewicz University in Poznań, Umultowska 89, Poznań, Poland.

Animals (measurements and statistics in Table 5): Body almost transparent in small specimens and whitish in adults; after fixation in Hoyer's medium body transparent (Fig. 7A). Eyes present in alive animals and dissolved by Hoyer's medium in approximately 90% of all mounted specimens. Body cuticle smooth, *i.e.*, without pores, body granulation or tubercles, but a fine, poorly visible network-like sculpture is present on the dorsal cuticle (Fig. 7D). A fine granulation present on the external surface of legs I-III (Fig. 7B), whereas on the internal surface the granulation is absent (Fig. 7C). Granulation is also present on the lateral and dorsal surfaces of legs IV (Fig. 7D). A cuticular bulge, similar to a pulvinus, is present on the internal surface of legs I-III (Fig. 7C). Claws of the Mesobiotus type, with a peduncle connecting the claw to the lunula, a



Fig. 6. Mesobiotus diegoi sp. nov. - reproduction: male with testis filled with spermatozoa. Scale bars in µm.

basal septum, and well-developed accessory points situated parallel to the primary branch (Fig. 8A–B). Lunulae under all claws smooth (Fig. 8A–B). A single continuous cuticular bar with shadowed extensions narrowing toward double muscle attachments is present below claws I–III (Figs. 7C, 8A), while a horseshoeshaped structure connects the anterior and posterior lunulae on claws IV (Fig. 8B).

Mouth antero-ventral. Bucco-pharyngeal apparatus of the *Macrobiotus* type (Fig. 9A), with ventral lamina and ten small peribuccal lamellae. The oral cavity armature well developed and composed of three bands of teeth (Fig. 9B–E). The first band of teeth is composed of numerous small granules arranged in several discrete rows situated anteriorly in the oral cavity, just behind the bases of the peribuccal lamellae (Fig. 9B–E). The second band of teeth is located between the ring fold and the third band of teeth and is composed of ridges parallel to the main axis of the buccal tube that are larger than those in the first band (Fig. 9B–E). The teeth of the third band are located within the posterior portion of the oral cavity, between the second band of teeth and the opening of the buccal tube (Fig. 9B–E). The third band of teeth is discontinuous and divided into a dorsal and ventral portion. Under PCM, dorsal and ventral teeth are visible as two lateral ridges /

Table 5. Measurements [in μ m] and pt values of selected morphological structures of animals of *Mesobiotus maklowiczi* sp. nov.; specimens mounted in Hoyer's medium

Character	N Range		Me	Mean		SD		Holotype	
		μm	pt	μm	pt	μm	pt	μm	pt
Body length	20	322-527		416		61		519	
Buccal tube									
Buccal tube length	20	36.3-51.6	_	41.4	_	4.7	_	47.1	_
Stylet support insertion point	20	27.3-39.5	75.0–76.7	31.4	76.0	3.6	0.6	35.9	76.2
Buccal tube external width	20	4.7-8.6	12.7–16.7	6.0	14.5	1.1	1.2	7.7	16.3
Buccal tube internal width	20	3.3-6.5	9.0–12.6	4.5	10.9	0.9	1.1	5.8	12.3
Ventral lamina length	18	23.0-33.1	60.7-67.4	26.2	63.9	3.0	2.0	28.6	60.7
Placoid lengths									
Macroplacoid 1	20	4.8-10.9	13.2–21.1	6.5	15.5	1.5	1.8	8.1	17.2
Macroplacoid 2	20	3.7-6.8	10.1–13.4	4.9	11.7	0.8	1.0	6.2	13.2
Macroplacoid 3	20	4.0-8.9	10.9–17.2	5.4	13.0	1.2	1.5	6.5	13.8
Microplacoid	20	2.3-4.2	6.3–8.8	3.1	7.4	0.5	0.6	3.5	7.4
Macroplacoid row	20	15.0-28.1	41.0–54.5	19.0	45.6	3.4	3.3	23.5	49.9
Placoid row	20	17.9-33.7	48.9–65.3	23.1	55.6	4.0	3.7	28.5	60.5
Claw I heights									
External primary branch	17	8.3-12.1	19.1–26.4	9.4	23.0	0.8	2.2	9.0	19.1
External secondary branch	17	6.2-10.6	16.1–23.7	7.8	18.9	1.0	2.4	7.7	16.3
Internal primary branch	16	8.1-11.8	18.9–25.6	9.2	22.3	0.9	2.2	8.9	18.9
Internal secondary branch	16	6.0-10.1	14.9–21.4	7.3	17.7	1.0	2.1	7.3	15.5
Claw II heights									
External primary branch	18	8.9-12.9	20.0-27.3	10.0	24.2	0.9	2.2	10.3	21.9
External secondary branch	18	7.2-10.7	15.9–24.5	8.3	20.1	0.8	2.4	7.9	16.8
Internal primary branch	18	8.2-12.8	19.5–27.1	9.5	23.0	1.0	2.5	9.7	20.6
Internal secondary branch	17	6.4–10.7	15.6–22.6	7.7	18.6	0.9	2.3	7.8	16.6
Claw III heights									
External primary branch	17	8.8-13.0	20.7–28.9	10.0	24.3	0.9	2.5	10.1	21.4
External secondary branch	16	7.2-10.8	15.7–25.1	8.2	20.0	0.9	2.6	8.9	18.9
Internal primary branch	17	8.5-12.4	19.4–28.1	9.7	23.4	0.8	2.3	10.0	21.2
Internal secondary branch	17	6.8-10.8	15.7–22.8	7.8	18.8	0.9	2.0	7.4	15.7
Claw IV heights									
Anterior primary branch	16	9.7-15.0	22.3–34.4	11.5	28.1	1.1	3.6	11.1	23.6
Anterior secondary branch	14	7.8-12.2	17.4–26.4	9.1	22.1	1.0	2.5	9.2	19.5
Posterior primary branch	17	10.6-15.9	25.8-35.5	12.4	30.0	1.3	2.8	13.2	28.0
Posterior secondary branch	11	8.2-12.3	20.8–27.5	9.9	23.0	1.2	2.2	10.9	23.1

N, number of specimens/structures measured. Range, refers to the smallest and the largest structure among all measured specimens; SD, standard deviation.



Fig. 7. *Mesobiotus maklowiczi* sp. nov. – PCM image of habitus, leg, and dorsal cuticle morphology: (A) dorso-ventral projection (holotype); (B) granulation on the external surface of leg II (holotype); (C) a pulvinus-like cuticular bulge on the internal surface of leg II (holotype); (D) granulation on the dorsal and lateral surface of leg IV and network-like sculpture in dorsal cuticle (holotype). Empty indented arrowheads indicate network-like sculpture in dorsal cuticle. Scale bar in µm.

crests and one median transverse ridge / crest (Fig. 9B– E). Sometimes, additional granular teeth are present between the second and third band of teeth (Fig. 9D–E) or even below the third band of teeth further toward the pharynx in the buccal tube (Fig. 9C). Pharyngeal bulb ovoid (Fig. 3A), with triangular apophyses, three rodshaped macroplacoids, and a drop-shaped microplacoid placed close to the third macroplacoid (Fig. 9F–G). The macroplacoid length sequence is 2 < 3 < 1. The first macroplacoid is anteriorly narrowed and the third has a clearly defined subterminal constriction (Fig. 9F–G).

Eggs (measurements and statistics in Table 6): White, laid free, spherical in shape and equipped with large, evenly spaced processes in the shape of wide cones with collars and with the distal part thinned and flexible (Figs. 10A-F, 11A-F). Egg surface between the processes without areolation. In PCM and in SEM the egg surface between processes comprises a system of irregularly distributed ridges and small pores between them (Figs. 10A-D, 11C-D). The structure may resemble reticulation; however, the ridges are mainly wider than pores diameter (pores diameter range: 0.2-0.6 µm; Figs. 10A-D, 11C-D). In SEM the ridges have a rough surface, but it cannot be excluded that this might be a preparation artifact or dirt (Fig. 11C-E). Basal part of the processes with well-developed collar elevated above the egg surface (Figs. 10A-D, 11A-E). From the top view the collar gives the impression that the processes base is slightly pentagonal in shape (Figs. 10A-D, 11A-E). Faint dark thickenings are present around the processes bases under the collar and visible only in PCM and only when the collar is folded back

(Fig. 10D). The labyrinthine layer is visible under PCM as a reticulum in the process walls, with varying mesh sizes uniformly distributed within the process walls (Fig. 10A–D). The walls of the processes are punctured with large pores (1.0–3.0 μ m in size) that are located around the base of the process just above the collar (Figs. 10A–D, 11A–F). The distal portion of the processes is usually strongly elongated and flexible, often containing internal bubble-like structures visible in the process midsection (Fig. 10F). The flexible upper portions of egg processes are smooth and not covered with granules (Fig. 11C–D), and these top portions rarely can be bi- or trifurcated. The egg surface under the processes is covered by small granulation, which can be visible only in SEM through the large pores perforating the process wall (Fig. 11F).

Reproduction: The new species is dioecious. Spermathecae filled with sperm have not been found in gravid females on freshly prepared slides. However, in males, the testes, filled with sperm, are clearly visible under PCM up to 48 hours after mounting in Hoyer medium (Fig. 12). The new species does not exhibit male secondary sexual dimorphism traits such as lateral gibbosities on legs IV.

DNA sequences:

The sequences obtained for only three out of all four molecular markers analysed in this study were of good quality and were represented by single haplotypes. Several attempts to amplify the ITS-2 marker for the new species failed, which prevented obtaining these sequences.

The 18S rRNA sequences (GenBank: OP142524, OP142525), 989 bp long;



Fig. 8. *Mesobiotus maklowiczi* sp. nov. – PCM images of claws: (A) claws II with smooth lunulae (paratype); (B) claws IV with smooth lunulae (paratype). Filled flat arrowhead indicates a single continuous cuticular bar below the claws, empty flat arrowheads indicate paired muscles attachments, filled indented arrowhead indicates horseshoe structure connecting the anterior and the posterior claw. Scale bars in µm.





Fig. 9. *Mesobiotus maklowiczi* sp. nov. – PCM images of the buccal apparatus: (A) an entire buccal apparatus (paratype); (B–C) the oral cavity armature, dorsal and ventral teeth respectively (bigger paratype); (D–E) the oral cavity armature, dorsal and ventral teeth respectively (smaller paratype); (F–G) placoid morphology, dorsal and ventral placoids respectively (paratype). Filled flat arrowheads indicate the first band of teeth, filled indented arrowheads indicate the third band of teeth, empty indented arrowheads indicate subterminal constrictions in the third macroplacoid. Scale bars in μm.



Fig. 10. *Mesobiotus maklowiczi* sp. nov. – PCM images of the egg. (A–D) egg surface; (E–F) egg processes midsections. Filled flat arrowheads indicate a collar surrounding the egg process, empty flat arrowheads indicate poorly visible crown of thickenings around the processes bases (visible only when the collar is upfolded), filled indented arrowheads indicate big pores puncturing egg process wall above the collar. Scale bars in µm.

Table 6.	Measurements	[in µm] o	of the egg	gs of <i>Mesobiotus</i>	maklowiczi	sp. nov.	, eggs	mounted	in Hoyer [*]	's medium;
process ba	ase/height ratio i	is expresso	ed as perc	centage						

Character	Ν	Range	Mean	SD
Egg bare diameter	20	59.1-81.0	69.3	6.0
Egg full diameter	20	98.4-133.8	118.7	10.3
Process height	60	20.0-34.8	25.9	3.5
Process base width	60	11.9-21.0	16.4	1.9
Process base/height ratio	60	39%-85%	64%	8%
Inter-process distance	60	2.6-5.5	4.1	0.7
Number of processes on the egg circumference	20	10-12	11.0	0.3

N, number of eggs/structures measured. Range, refers to the smallest and the largest structure among all measured specimens. SD, standard deviation.



Fig. 11. *Mesobiotus maklowiczi* sp. nov. – SEM images of eggs: (A–B) entire view of the egg; (C–D) egg processes; (E) details of the egg surface between processes; (F) big pore puncturing egg process wall above the collar. Filled flat arrowheads indicate a collar surrounding the egg process, filled indented arrowheads indicate big pores puncturing egg process wall above the collar. Scale bars in µm.

The 28S rRNA sequences (GenBank: OP142518, OP142519), 759 bp long;

The *COI* sequences (GenBank: OP143855, OP143856), 678 bp long.

Phylogenetic results

Both phylogenetic analyses resulted with trees of similar topology and well-supported nodes in which a monophyletic and paraphyletic clades of non-Antarctic and Antarctic taxa can be distinguished, respectively (Fig. 13). The phylogenetic investigation did not recover the M. harmsworthi and M. furciger morpho-groups to be monophyletic since the respective representatives of these two groups are intermixed in the presented phylogeny (Fig. 13). Both new species described in this study clustered together with other Mesobiotus taxa from tropical and subtropical regions. The analysis recovered Mesobiotus maklowiczi sp. nov. to be the closest relative of Mesobiotus anastasiae Tumanov, 2020 (Fig. 13). While Mesobiotus diegoi sp. nov. stays in sister relationship to the whole clade comprising tropical and subtropical species, namely: Mesobiotus imperialis Stec, 2021, Mesobiotus philippinicus Mapalo, Stec, Mirano-Bascos and Michalczyk, 2016, Mesobiotus fiedleri Kaczmarek, Bartylak, Stec, Kulpa, M. Kepel, A. Kepel and Roszkowska, 2020, M. maklowiczi sp. nov., M. anastasiae, Mesobiotus radiatus (Pilato, Binda and Catanzaro, 1991), Mesobiotus ethiopicus Stec and Kristensen, 2017, Mesobiotus datanlanicus Stec, 2019, Mesobiotus insanis Mapalo, Stec, Mirano-Bascos and Michalczyk, 2017, Mesobiotus romani Roszkowska, Stec, Gawlak and Kaczmarek, 2018. In this study, newly analysed M. peterseni is in a sister relationship with the other three taxa, namely: M. harmsworthi, Mesobiotus occultatus Kaczmarek, Zawierucha, Buda,

Stec, Gawlak, Michalczyk and Roszkowska, 2018 and unspecified species of the *M. harmsworthi* morphogroup from Russia. The Antarctic part of the tree comprises six monophyletic clades akin to species, but only *Mesobiotus hilariae* Vecchi, Cesari, Bertolani, Jönsson, Rebecchi and Guidetti, 2016 represents a formally named taxon.

DISCUSSION

Differential diagnosis of *Mesobiotus diegoi* sp. nov.

The new species belongs to the informal *Mesobiotus harmsworthi* morpho-group as it exhibits rather large conical processes. By having (*i*) extremely long conical processes that are higher than 25 μ m as well as (*ii*) egg surface without areolation and finger-like projections, the new species is similar to the following taxa: *Mesobiotus altitudinalis* (Biserov, 1997/98), *Mesobiotus joenssoni* Guidetti, Gneuß, Cesari, Altiero and Schill, 2020, and *M. radiatus*, but it differs specifically from:

Mesobiotus altitudinalis, known only from Russia (North Ossetia, the Caucasus Mts; Biserov 1997/98), by the presence of eyes (eyes absent in *M. altitudinalis*), the robust claws on all legs with two branches diverging at half of the total claw length (the claws with elongated branches that diverge at 1/3 of the total claw length in *M. altitudinalis*), the presence of cuticular bars in legs I–III (the cuticular bars absent *M. altitudinalis*), the presence of a ring of large meshes of the labyrinthine layer at the processes bases (the ring of large meshes absent in *M. altitudinalis*), the presence of dark thickenings around the egg processes bases (the thickenings absent



Fig. 12. Mesobiotus maklowiczi sp. nov. - reproduction: male with testis filled with spermatozoa. Scale bars in µm.

in *M. altitudinalis*), the presence of evident bubble-like structure within the distal portion of the egg processes.

Mesobiotus joenssoni, known only from Italy (the island of Elba; Guidetti et al. 2020), by: the absence of small tubercles in the dorsal and dorsolateral cuticle in the posterior part of the body (the tubercles present in *M. joenssoni*; the authors call this structure granules, whereas its very distinct and larger from leg and body granulation, which is typically reported for macrobiotid taxa. Therefore, the term 'tubercles' should be less confusing when describing this structure), the presence of granulation on the internal leg surface in legs I–III (the internal granulation absent in *M. joenssoni*), the presence of slightly dentate lunulae in the hind legs (the lunulae in *M. joenssoni*), the absence of a collar

at the base of the egg processes (the collar present in *M. joenssoni*), the absence of large pores perforating the wall of the egg processes (the large pores present just above the collar in *M. joenssoni*), the presence of a ring of large meshes of the labyrinthine layer at the bases of the processes (the ring of large meshes absent in *M. joenssoni*), the presence of bubble-like structures within the distal, elongated portion of egg processes (the bubble-like structures absent in *M. joenssoni*).

Mesobiotus radiatus, known only from Tanzania, Kenya, and the Democratic Republic of Congo (Pilato et al. 1991; Binda et al. 2001; Stec et al. 2018b), by: the presence of eyes (eyes absent in *M. radiatus*), the presence of granulation on the internal leg surface in legs I–III (the internal granulation absent in *M.*



Fig. 13. Maximum likelihood (ML) phylogeny constructed from concatenated sequences (18S rRNA + 28S rRNA + ITS-2 + COI) of the genus *Mesobiotus*. Numbers above branches indicate bootstrap support values, while Bayesian posterior probabilities (pp) are given below branches. Bootstrap < 70 and pp < 0.95 are not shown. The taxa newly sequenced in this study are marked with bolded font. Taxa of the *M. harmsworthi*, *M. furciger*, and *M. montanus* morpho-groups are indicated by blue, red, and green font, respectively. The outgroup is indicated in gray font. The scale bar represents substitutions per position.

radiatus), the presence of a large median tooth in the ventral portion of the third band of teeth in the oral cavity armature (OCA; the ventro-median tooth divided into 2-4 round median teeth in *M. radiatus*), the absence of spurs at the claw bases (short and very thin spurs are often present in *M. radiatus*), the presence of a ring of large meshes of the labyrinthine layer at the bases of the processes (the ring of large meshes absent in M. radiatus), evidently smaller pores in the distal portion of the egg processes $(0.15-0.20 \ \mu m$ in diameter in the new species vs. 0.30-0.60 µm in diameter in M. radiatus; the range for M. radiatus confirmed de novo based on the figures in Stec et al. (2018b)), the absence of a bundle of short flexible filaments at the egg processes apieces (the filaments present in *M. radiatus*), the absence of micro granulation at the most distal portion of egg processes (flexible filaments covered with micro granulation in M. radiatus), a larger egg full diameter (141.0-178.9 µm in the new species vs. 97.8-131.1 µm in M. radiatus), a slightly larger egg process height (30.7-47.3 µm in the new species vs. 15.5–29.3 µm in M. radiatus).

Remarks: the comparison was made using data on *M. radiatus* presented by Stee et al. (2018b).

Differential diagnosis of *Mesobiotus maklowiczi* sp. nov.

The new species belongs to the informal *Mesobiotus harmsworthi* morpho-group as it exhibits rather large conical processes. By having egg processes in the shape of sharp wide cones with collar, the new species is similar to the following taxa: *M. anastasiae*, *M. joenssoni*, and *Mesobiotus mauccii* (Pilato, 1974), but it differs specifically from:

Mesobiotus anastasiae, known only from the Republic of South Africa (Tumanov 2020), by: the presence of granulation on the internal leg surface in legs I–III (the internal granulation absent in M. anastasiae), a different morphology of the first band of teeth in the OCA (the first band consists of several rows of small granular teeth in the new species vs. the first band consist of one row of granular teeth in *M. anastasiae*), a different morphology of the second band of teeth in the OCA (the second band composed of ridges parallel to the main axis of the buccal tube, sometimes with supplementary teeth between the in the new species – harmsworthi type sensu Kaczmarek et al. (2020) vs. the second band consists of several rows of granular teeth in M. anastasiae - krynauwi type sensu Kaczmarek et al. (2020)), the presence of singular undivided ventro-median tooth in the third band of teeth in the OCA (the ventro-median tooth divided into two roundish teeth in *M. anastasiae*), the absence of larger pores below the collar at the base of egg processes (the larger pores present below the collar in *M. anastasiae*), a different morphology of the egg surface between processes (surface with a system of irregularly distributed ridges and densely distributed small pores between them, resembling reticulation in the new species vs. surface evidently porous with much less frequently spaced pores in *M. anastasiae*).

Mesobiotus joenssoni by: the presence of a reticulate pattern in the dorsal cuticle visible in PCM (the reticulate pattern absent in *M. joenssoni*), the absence of small tubercles in the dorsal and dorsolateral cuticle in the posterior part of the body (the tubercles present in *M. joenssoni*), the presence of granulation on the internal leg surface in legs I–III (the internal granulation absent in *M. joenssoni*), the presence of bubble-like structures within the distal elongated portion of egg processes (the bubble-like structures absent in *M. joenssoni*).

Mesobiotus mauccii, known from China (Pilato 1974; Beasley and Miller 2007 2012), South Andaman Island (Maucci and Durante Pasa 1980) and Japan (Utsugi 1988; Abe and Takeda 2000 2005), by the presence of a reticulate pattern in the dorsal cuticle visible in PCM (the reticulate pattern absent in *M. mauccii*), a narrower buccal tube (external buccal tube width is $4.7-8.6 \mu m$ in the new species vs. ca. 11 μm in *M. mauccii*), the absence of ridges on the egg surface between the processes, forming polygonate cells circling each process (the ridges present in *M. mauccii*).

Mesobiotus phylogeny and species composition

Similarly to other macrobiotid genera, the genus Mesobiotus also exhibits a rather stable and conservative animal morphology. Interestingly, egg morphotypes known in the genus exhibit one of the most drastic examples of morphological diversity in egg ornamentation (Kaczmarek et al. 2020; Stec et al. 2021). This is in line with the observation that chorion ornamentation evolves faster than animal morphology (Guidetti et al. 2013) which was also confirmed by experimental findings about the congruence between genetic and morphological divergence (Stec et al. 2016b). The increase in tempo in the morphological divergence of the egg chorion in tardigrades could be explained by two alternative hypotheses with strong or relaxed natural selection, respectively. In the first scenario, different morphotypes might be shaped by biotic and abiotic constraints and as such constitute adaptations to oviposition in different microhabitats that potentially increase protection and/or attachment properties but also ease dispersion as the empty processes make the egg lighter. Alternatively, if specific ornamentations do not have any adaptive value, relaxed natural selection would enable unconstrained and more flexible evolution that would derive a plethora of morphotypes. Currently, there is a lack of sufficient data to test these hypotheses properly.

As mentioned in the Introduction, *Mesobiotus* was established by integrative analysis of two former species complexes in the genus *Macrobiotus*, the *harmsworthi* and *furciger* groups (Vecchi et al. 2016), and the monophyly of the genus was subsequently confirmed by other studies (Guil et al. 2019; Stec et al. 2021). However, these informal species groups could not be accommodated into any subgeneric rank, as their representatives do not form monophyletic clades and are scattered in different places in the genus phylogeny (Kaczmarek et al. 2018 2020; Stec 2021, Stec et al. 2021 2022; Short et al. 2022; this study). The two informal groups within the genus are recognized elusively by egg morphology and were recently criticized by Short et al. (2022), who demonstrated

large divergence between Antarctic and non-Antarctic Mesobiotus taxa and proposed to abandon the usage of informal groups within the genus. The authors reasoned that the groups have no systematic value as they are not monophyletic clades and as such, they hide evolutionary relationships and biogeographical patterns. However, I argue that (i) given the extreme morphological diversity within the genus, informal groups have a tremendous practical value for both taxonomists and name-users in aiding navigation, identification, and communication regarding taxa, and (ii) the confusion regarding these informal groups results most likely from their elusive working definitions. Therefore, to clarify the distinction between the aforementioned informal species groups, I here propose explicit criteria coming from egg morphological characters that should be met to include Mesobiotus taxa to species morpho-groups. This action resulted in the creation of the third informal taxonomic



Fig. 14. *Mesobiotus peterseni* (Maucci, 1991) from Greenaland – PCM images of the egg. (A–B) entire egg with egg processes midsections seen on the egg circumference; (C–E) egg surface. Scale bars in µm.

group of species that greatly differ from the *furciger* and *harmsworthi* egg morphotype (Fig. 14). I propose to use the term "morpho-groups" when referring to those divisions that, according to Stec et al. (2021), should represent nonmonophyletic sets of phenotypically similar taxa. *Mesobiotus* morpho-groups are as follows:

Mesobiotus furciger morpho-group

Egg processes in the shape of branched cones, processes with smooth walls (without labyrinthine layer that is seen as reticulation) or with light refracting areas.

Group composition

Mesobiotus furciger (Murray, 1907b), Mesobiotus pilatoi (Binda and Rebecchi, 1992), Mesobiotus fiedleri Kaczmarek, Bartylak, Stec, Kulpa, M. Kepel, A. Kepel and Roszkowska, 2020, Mesobiotus marmoreus Stec, 2021, Mesobiotus siamensis (Tumanov, 2006), Mesobiotus divergens (Binda, Pilato and Lisi, 2005), Mesobiotus dilimanensis Itang, Stec, Mapalo, Mirano-Bascos and Michalczyk, 2020, Mesobiotus creber (Pilato & Lisi, 2009), Mesobiotus orcadensis (Murray, 1907c), Mesobiotus aradasi (Binda, Pilato and Lisi, 2005), Mesobiotus sicheli (Binda, Pilato and Lisi, 2005).

Mesobiotus montanus morpho-group

Egg processes in the shape of hemispherical or mammillate-like domes.

Group composition

Mesobiotus montanus (Murray, 1910), Mesobiotus mottai (Binda and Pilato, 1994), Mesobiotus peterseni (Maucci, 1991), Mesobiotus lusitanicus (Maucci and Durante Pasa, 1984)*.

**Remarks: Mesobitous lusitanicus* exhibits a considerable variation in the morphology of the egg processes. However, it is included in the *M. montanus* morpho-group as the typical form of the processes is mammillate-like domes. The abnormal form of egg reported in the original description may actually belong to a different *Mesobiotus* species, which could have also been present in the analysed samples.

Mesobiotus harmsworthi morpho-group

Egg processes in the shape of cones with diverse morphology of process endings (long slender endings, long slender endings with filaments, sharp endings, endings with flexible filaments, truncated endings).

Groups composition

Mesobiotus altitudinalis (Biserov, 1997/98), Mesobiotus anastasiae Tumanov, 2020, Mesobiotus arguei (Pilato and Sperlinga, 1975), Mesobiotus armatus (Pilato and Binda, 1996) [nomen inquirendum], Mesobiotus australis (Pilato and D'Urso, 1976), Mesobiotus baltatus (McInnes, 1991), Mesobiotus barabanovi (Tumanov, 2005), Mesobiotus barbarae (Kaczmarek, Michalczyk and Degma, 2007), Mesobiotus binieki (Kaczmarek, Gołdyn, Prokop and Michalczyk, 2011), Mesobiotus blocki (Dastych, 1984), Mesobiotus contii (Pilato and Lisi, 2006b), Mesobiotus coronatus (de Barros, 1942), Mesobiotus datanlanicus Stec, 2019, Mesobiotus diffusus (Binda and Pilato, 1987), Mesobiotus diguensis (Pilato and Lisi, 2009), Mesobiotus dimentmani (Pilato, Lisi and Binda, 2010), Mesobiotus emiliae Massa, Guidetti, Cesari, Rebecchi and Jönsson, 2021, Mesobiotus erminiae (Binda and Pilato, 1999), Mesobiotus ethiopicus Stec and Kristensen, 2017, Mesobiotus harmsworthi (Murray, 1907a), Mesobiotus helenae Tumanov and Pilato, 2019, Mesobiotus hieronimi (Pilato and Claxton, 1988), Mesobiotus hilariae Vecchi, Cesari, Bertolani, Jönsson, Rebecchi and Guidetti, 2016, Mesobiotus imperialis Stec, 2021, Mesobiotus insanis Mapalo, Stec, Mirano-Bascos and Michalczyk, 2017, Mesobiotus insuetus (Pilato, Sabella and Lisi, 2014), Mesobiotus joenssoni Guidetti, Gneuß, Cesari, Altiero and Schill, 2020, Mesobiotus kovalevi (Tumanov, 2004), Mesobiotus krynauwi (Dastych and Harris, 1995), Mesobiotus liviae (Ramazzotti, 1962), Mesobiotus mauccii (Pilato, 1974), Mesobiotus meridionalis (Richters, 1909) [nomen inquirendum], Mesobiotus neuquensis (Rossi, Claps and Ardohain, 2009), Mesobiotus nikolaevae Tumanov, 2018, Mesobiotus nuragicus (Pilato and Sperlinga, 1975), Mesobiotus occultatus Kaczmarek, Zawierucha, Buda, Stec, Gawlak, Michalczyk and Roszkowska, 2018, Mesobiotus ovostriatus (Pilato and Patanè, 1998), Mesobiotus patiens (Pilato, Binda, Napolitano and Moncada, 2000), Mesobiotus perfidus (Pilato and Lisi, 2009), Mesobiotus philippinicus Mapalo, Stec, Mirano-Bascos and Michalczyk, 2016, Mesobiotus polaris (Murray, 1910) [nomen inquirendum], Mesobiotus pseudoblocki Roszkowska, Stec, Ciobanu and Kaczmarek, 2016, Mesobiotus pseudocoronatus (Pilato, Binda and Lisi, 2006), Mesobiotus pseudoliviae (Pilato and Binda, 1996), Mesobiotus pseudonuragicus (Pilato, Binda and Lisi, 2004), Mesobiotus pseudopatiens Kaczmarek and Roszkowska, 2016, Mesobiotus radiatus (Pilato, Binda and Catanzaro, 1991), Mesobiotus reinhardti (Michalczyk and Kaczmarek, 2003), Mesobiotus rigidus (Pilato and Lisi, 2006a), Mesobiotus romani Roszkowska, Stec, Gawlak and Kaczmarek,

2018, Mesobiotus simulans (Pilato, Binda, Napolitano and Moncada, 2000), Mesobiotus skorackii Kaczmarek, Zawierucha, Buda, Stec, Gawlak, Michalczyk and Roszkowska, 2018, Mesobiotus snaresensis (Horning, Schuster and Grigarick, 1978), Mesobiotus stellaris (du Bois-Reymond Marcus, 1944) [nomen inquirendum], Mesobiotus szeptyckii (Kaczmarek and Michalczyk, 2009), Mesobiotus tehuelchensis (Rossi, Claps and Ardohain, 2009), Mesobiotus wuzhishanensis (Yin, L. Wang and X. Li, 2011), Mesobiotus zhejiangensis (Yin, L. Wang and X. Li, 2011).

Updated key to species of Mesobiotus

Here, I provide an updated key following its previous versions published by Kaczmarek et al. (2020) and Tumanov (2020). For schematic drawings and figures depicting specific morphological structures mentioned/used in the key, please check Kaczmarek et al. (2020). The following five species were added to the key: M. emiliae, M. imperialis, M. marmoreus, Mesobiotus diegoi sp. nov., Mesobiotus maklowiczi sp. nov. The following four species were not included due to their designation as nomina inquirenda by Kaczmarek et al. (2020): Mesobiotus meridionalis (Richters, 1909) nom. inq., M. polaris (Murray, 1910) nom. inq., M. stellaris (du Bois-Reymond Marcus, 1944) nom. inq. and M. armatus (Pilato and Binda, 1996) nom. inq. The presented version comprises 71 nominal Mesobiotus species.

1. Dorsal cuticle with sculptured surface (visible in PCM as granulation or thin reticulate pattern) or with pores or stripes of Dorsal cuticle smooth (except for minute regular granulation visible only in SEM) 12 2. Stripes of pigmentation present (visible in animals freshly mounted in microscope slides) M. baltatus (McInnes, 1991) 3. First band of teeth in oral cavity present 4 First band of teeth in oral cavity absent or not visible in PCM M. perfidus (Pilato and Lisi, 2009) 4 Cuticle with singular pores, without granulation on body surface or on legs M. krynauwi (Dastych & Harris, 1995) Cuticle with sculpture, visible in PCM as granulation or dot-like sculpture, or with thin reticulate pattern, without pores 5 Cuticular sculpture consists of relatively large granules/tubercles, 5. well-visible in PCM (granules size $\geq 1 \ \mu m$) in caudal region of Cuticular sculpture without large granules/tubercles, with fine dot-like sculpture poorly visible in LM or with thin reticulate 6. Granules/tubercles of cuticular sculpture are present from the level of third legs to posterior end of animal, pt of stylet supports 72.41-83.64, egg processes 27-36 µm high, with collar and large pores above it M. joenssoni Guidetti, Gneuß, Cesari, Altiero & Schill, 2020 Granules/tubercles of cuticular sculpture are present only on caudal extremity of body, pt of stylet supports ca 85.4, maximal height of egg processes is 19 μ m, egg processes without collar and large pores above it *M. arguei* (Pilato and Sperlinga, 1975)

- Oral cavity armature with longitudinally elongated teeth in second band, with several row of granular teeth in first band, granulation on internal leg surface in legs I–III present, pores present only above collar in egg process wall
- Egg shell surface porous
 M. sicheli (Binda, Pilato and Lisi, 2005)

- Lunulae IV with indented margin, eyes present, areolation on egg surface absent, egg process bases with crown of thickenings
 M. pseudocoronatus (Pilato, Binda and Lisi, 2006)
- Egg shell surface with reticular sculpture, egg processes bases without crown of thickenings *M. montanus* (Murray, 1910)
- Egg shell surface without reticular sculpture, egg processes bases with crown of thickenings and wrinkles
- 14. Egg processes "mammillate-like domes"
 15

 Egg processes in shape of cones
 16

- 17.
 Egg processes in shape of truncated cones
 17

 17.
 Egg processes in shape of truncated cones
 18

 17.
 Egg processes different
 18

 18.
 Egg processes bases elongated into long stripes that form the arcolation on egg shell surface (full arcolation)
 19

 Full arcolation on egg shell surface absent
 27

 19.
 First band of teeth in oral cavity absent
 20
- First band of teeth in oral cavity present 21

20.	Lunulae IV smooth, egg processes in shape of cones with long
_	slender endings
	M. hilariae
	Vecchi, Cesari, Bertolani, Jøonsson, Rebecchi and Guidetti, 2016
21.	Egg processes with terminal filaments
- 22.	Eyes absent, macroplacoid length sequence $(2 \le 1 \le 3)$, process
	apices divided into at least 15 filaments
	<i>M. insanis</i> Mapalo, Stec, Mirano-Bascos and Michalczyk, 2017
-	Eyes present, macroplacoid length sequence $(2 < 1 = 3)$, process
	apices divided into 2–5 filaments
23.	Egg processes are usually terminated by a multifurcated crown
	of several finger-shaped appendages, often terminated by short
	spines M. datanlanicus Stec, 2019
-	Egg processes without crown of appendages at the top
24.	Egg processes in shape of cones with long slender endings
-	Egg processes sharp, narrow or wide cones
25.	Granulation on legs absent, egg processes in shape of sharp wide
	cones, height of egg processes less than 16.0 µm
-	uranulation on legs present, egg processes in shape of sharp
26.	The <i>pt</i> of stylet supports less than 75.0 , six areoles around each
	egg process, height of egg processes less than 35.0 μ m, width of
	egg processes bases less than 22.0 µm
	<i>M. hieronimi</i> (Pilato and Claxton, 1988)
-	each egg process height of egg processes more than 41.0 um
	width of egg processes bases more than 27.0 µm
27.	Egg processes bases elongated into long stripes, which form
	semi-areolation (stripes/ridges at least in some cases not
_	Equip processes bases different 32
28.	Eyes absent, egg processes with short flexible filaments
-	Eyes present, egg processes without filaments 29
29.	Additional teeth in oral cavity armature present, egg processes
-	Additional teeth in oral cavity armature absent egg processes
	without bubble-like structures
30.	Claws IV with large, protruding accessory points, egg processes
	with reticular design, number of processes on egg circumference
	11–12 <i>M. harmsworthi</i> (Murray, 1907a)
-	Large and protrucing accessory points on claws IV absent, egg processes without reticular design number of processes on egg
	circumference 15–24 <i>M. blocki</i> (Dastych. 1984)
31.	Granulation on legs I-III absent, egg processes in shape of
	cones with long slender endings, number of processes on egg
	circumference ca 20, claws with evidently elongated branches
_	Granulation on legs I-III present and processes in shape of share
-	wide cones, number of processes on egg circumference $10-12$.
	normal <i>Mesobiotus</i> type claws without elongated branches
22	Zawierucha, Buda, Stec, Gawlak, Michalczyk and Roszkowska, 2018
32.	Egg processes with finger-like projections, <i>i.e.</i> , egg processes
	other
-	Egg processes without finger-like projections
22	

-	Egg processes in the shape of sharp, narrow or wide cones 36
34.	Egg processes with bubble-like structures and flexible filaments
	in apical part, height of egg processes less than 13.0 µm
	<i>M. pseudoblocki</i> Roszkowska, Stec, Ciobanu and Kaczmarek, 2016
-	Egg processes without with bubble-like structures or flexible
	filaments in apical part, height of egg processes more than
	20.0 μm
35.	Eves present, number of processes on egg circumference $6-8$.
	height of eag processes 50.0 µm or more
	<i>M liviae</i> (Demozratii 1062)
	M. uvue (Rainazzoui, 1902)
-	Eyes absent, number of processes on egg circumference ca 12,
	height of egg processes ca 21.0 µm
	<i>M. snaresensis</i> (Horning, Schuster and Grigarick, 1978)
36.	Egg processes in shape of sharp narrow cones and with bubble-
	like structures
-	Egg processes in shape of sharp wide cones and without bubble-
	like structures
37	Eves absent additional teeth in oral cavity absent
57.	M tahualchansis (Rossi Claps and Ardobain 2009)
	Even messent additional teath in and acvity messent
-	Eyes present, additional teeth in oral cavity present
38.	Finger-like projections poorly marked, present only in some egg
	processes and irregularly distributed
	<i>M. reinhardti</i> (Michalczyk and Kaczmarek, 2003)
-	Six, large and regularly distributed finger-like projections present
	in all egg processes
	M. szeptyckii (Kaczmarek and Michalczyk, 2009)
39.	Eves and first band of teeth in oral cavity present. <i>pt</i> of stylet
	supports 77.0 or more, egg full diameter (with processes) 100.0–
	116.0 um number of processes on egg circumference 8–9 height
	of eag processes 12 0–24 0 µm
	M erminiae (Binda and Pilato 1999)
_	Eves absent first band of teeth in oral cavity absent or not visible
	in PCM at of stylet supports 75.5 or less egg full diameter
	(with processes) 88.0.02.0 µm number of processes on age
	(with processes) 88.0–92.0 µm, humber of processes on egg
	circumerence $12-13$, neight of egg processes $9.0-11.0 \ \mu m$
10	<i>M. alguensis</i> (Pilato and Lisi, 2009)
40.	Egg processes with reticular design caused by labyrinthine layer
-	Egg processes smooth or with refracting areas
41.	First band of teeth in oral cavity absent or not visible in PCM
	M. pseudopatiens Kaczmarek and Roszkowska, 2016
-	First band of teeth in oral cavity present
42.	Egg processes with terminal filaments (at least a significant part
	of them)
_	Egg processes without terminal filaments 50
43	Egg processes in share of sharp wide cones 44
чу.	Egg processes in shape of some with long slander andings
-	Egg processes in shape of cones with fong, siender endings 47
44.	Egg processes with lew long illaments (usually longer than
	5 µm), egg shell surface with reticular design
	<i>M. dimentmani</i> (Pilato, Lisi and Binda, 2010)
-	Egg processes with short filaments (usually shorter than 5 μ m),
	egg shell surface porous or with faintly light refracting dots (seen
	in PCM)
45.	Eyes absent, egg processes with bunch of short filaments, number
	of processes on egg circumference 10-12
-	Eyes present, egg processes with only few short filaments.
	number of processes on egg circumference 15–18 46
46.	Granulation on legs I–III visible in light microscope. well
	pronounced crown of thickenings around egg processes bases.
	unevenly distributed depressions and faint tubercles in ego
	processes walls (observable only in SEM)
	M imperialis Step 2021
	1911 impertation 5000, 2021

33. Egg processes in shape of cones with long slender endings 34

- Granulation on legs I-III not visible in light microscope, poorly

pronounced crown of thickenings around egg processes bases, philippinicus Mapalo, Stec, Mirano-Bascos and Michalczyk, 2016

- 47. Lunulae IV without indentation .. M. nikolaevae Tumanov, 2018
- 48. Egg processes bases without distinct crown of thickenings, processes are connected with very thin ridges
- M. diffusus (Binda and Pilato, 1987) Egg processes bases with distinct crown of thickenings, egg shell surface between processes with dots and wrinkles 49
- 49. Eyes present, lunulae IV with 4-5 denticles, egg processes tips sometimes trifurcated M. wuzhishanensis (Yin, Wang and Li, 2011)
- Eyes absent, lunulae IV with more than 5 denticles, egg processes tips never trifurcated M. romani Roszkowska, Stec, Gawlak and Kaczmarek, 2018
- 50. Egg processes in the shape of sharp wide cones 51
- Egg processes in the shape of cones with long, slender endings ...
- 51. Lunulae IV indented M. simulans (Pilato, Binda, Napolitano and Moncada, 2000)
- 52. Additional teeth in oral cavity present, egg bare diameter (without processes) 55.0 or less, egg full diameter (with processes) 71.0 µm or less, width of egg processes bases less than 10.5
- Additional teeth in oral cavity absent, egg bare diameter (without processes) 59.0 or more, egg full diameter (with processes) 73.0 µm or more, width of egg processes bases 11.1 or more ... 53
- 53. The basal tract of posterior and anterior claws IV much longer, primary and secondary branches forming an almost 90° angle (Pilato et al., 2014: Fig. 1d)
- M. insuetus (Pilato, Sabella and Lisi, 2014) Typical Mesobiotus claws IV 54
- 54. Granulation on legs I-III not visible in light microscope M. emiliae Massa, Guidetti, Cesari, Rebecchi and Jönsson, 2021
- Granulation on legs I-III well visible in light microscope 55 55. Eyes absent, macroplacoid length sequence (2 < 3 < 1), additional
- teeth in oral cavity absent M. patiens (Pilato, Binda, Napolitano and Moncada, 2000)
- Eyes present, macroplacoid length sequence $(2 < 3 \le 1)$, with additional teeth in the ventral portion of oral cavity M. occultatus Kaczmarek, Zawierucha, Buda, Stec, Gawlak, Michalczyk & Roszkowska, 2018
- 56. Egg shell surface porous, height of egg processes 22.0 µm or more, width of egg processes 17.0 µm or more 57
- Egg shell surface smooth or with dots and/or wrinkles, height of egg processes 16.5 µm or less, width of egg processes 15.5 µm or
- 57. Claw branches diverging at 1/3 of the total claw length, egg processes bases without crown of thickenings, distal portion of egg processes without bubble-like structure, ring of large meshes in process reticulum (labyrinthine layer) at processes base absent, cuticular bars in legs I-III absent
 - M. altitudinalis (Biserov, 1997/98)
- Claw branches diverging at half of the total claw length, egg processes bases with crown of thickenings, distal portion of egg processes with evident bubble-like structure, ring of large meshes in process reticulum (labyrinthine layer)at processes base present, cuticular bars in legs I-III present M. diegoi sp. nov.
- 58. Egg processes consists of wide short conical basal part very distinctly separated from apical part in form of a thin long spine with poorly visible internal structure, number of processes on egg circumference 27-32
 - .. M. binieki (Kaczmarek, Gołdyn, Prokop and Michalczyk, 2011)

- Egg processes with less abruptly separated basal and apical parts, apical part with well visible internal bubble-like structures, number of processes on egg circumference less than 23 59
- 59. Number of processes on egg circumference ca 12, height of egg processes 15.2–16.2 µm, with well visible reticular design in egg processes (labyrinthine layer), apical parts of egg processes rigid and never subdivided, egg shell surface with ridges radiating from processes bases M. rigidus (Pilato and Lisi, 2006b) Number of processes on egg circumference ca 22, height of egg processes 11.0 µm, with faint and almost invisible reticular design in egg processes (labyrinthine layer), apical parts of egg processes flexible and rarely bifurcated, egg shell surface smooth M. helenae Tumanov and Pilato, 2019 60. Egg processes in the shape of rough cones, egg processes base smooth M. kovalevi (Tumanov, 2004) Egg processes in the shape of branched or sharp wide cones, egg processes base with crown of thickenings or wrinkles 61 61. Egg processes in shape of sharp wide cones, egg shell surface without pores or reticular sculpture M. australis (Pilato and D'Urso, 1976) Egg processes in shape of branched cones, egg shell surface Egg processes without refracting areas 65 63. Bases of egg processes without band of pores, large and numerous refracting areas visible on apical part of all processes Bases of egg processes with band of pores, small and single refracting areas present only on some processes 64 64. Apical parts of egg processes always divided into 2-4 branches, height of egg processes ca 15.0 µm, pt of buccal tube width 22.8–25.4 M. pilatoi (Binda and Rebecchi, 1992) At least some of apical part of egg processes not divided, height of egg processes 8.5-13.1 µm, pt of buccal tube width 16.5-18.6 M. fiedleri Kaczmarek, Bartylak, Stec. Kulna, M. Kepel, A. Kepel and Roszkowska, 2020 65. Egg shell surface with reticular sculpture or ridges, without pores Egg shell surface porous 70 66. Egg processes in shape of branched cones with long slender endings, egg shell surface with ridges radiating from process Egg processes in shape of branched cones, egg shell surface without ridges radiating from process bases with clear reticular 67. Granulation present on all legs, lunulae IV smooth, stout processes with smooth trunks and apices divided into multiple slender, tentacular arms M. marmoreus Stec, 2021 Granulation absent on all legs, lunulae IV slightly indented, bottle-shaped processes with an evidently elongated distal part that is subdivided at the top into short and pointed apices 68. The pt of stylet supports less than 76.5, egg processes sparsely distributed over egg surface, number of processes on egg circumference ca 17, egg processes with relatively long branches, nearly equal in length to basal part, with multiple bifurcations, height of egg processes 4.7-4.8 µm M. divergens (Binda, Pilato and Lisi, 2005) The *pt* of stylet supports more than 77.0, egg processes densely distributed over egg surface, more than 17 processes on egg
- circumference, egg processes with relatively short branches, distinctly shorter than basal part, height of egg processes usually exceeds 4.8 µm 69
- 69. Additional teeth in oral cavity absent, granulation on legs I-III present, pt of buccal tube external width 14.0-17.4, number of

CONCLUSIONS

In this work, two new Mesobiotus species were identified using an integrative approach that combined morphological and morphometric data with genetics and phylogenetic information. Additionally, genetic data for Mesobiotus peterseni are presented for the first time. The multilocus molecular phylogeny elucidated the phylogenetic positions of newly studied taxa. It also confirmed the presence of deep evolutionary division of the genus into Antarctic and non-Antarctic taxa that also do not recover monophyly for the traditionally recognized informal species groups. The phylogeny, morphological diversity, and species composition of the genus were discussed in detail resulting in the distinction and ratification of three different morphogroups namely: M. furciger morpho-group, M. montanus morpho-group, M. harmsworthi morpho-group. This action should improve communication and navigation in future taxonomic studies on this diverse group of limno-terrestrial tardigrades. Finally, the updated key to all valid species of the genus Mesobiotus is provided above, in order to ease their identification.

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Supplementary materials

SM. 1. Raw morphometric data for *Mesobiotus diegoi* sp. nov. (download)

SM. 2. Raw morphometric data for *Mesobiotus maklowiczi* sp. nov. (download)

SM. 3. Model selections. (download)