Molecular Phylogenetics and Evolution 160 (2021) 106987

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

New multilocus phylogeny reorganises the family Macrobiotidae (Eutardigrada) and unveils complex morphological evolution of the *Macrobiotus hufelandi* group

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ARTICLE INFO

Keywords:
Integrative taxonomy
Molecular phylogeny
Morphological evolution
Sisubiotus **gen. nov.**
Xerobiotus
Tardigrada

ABSTRACT

The family Macrobiotidae is one of the most speciose and diverse groups among tardigrades. Although there have been attempts to reconstruct the phylogeny of this family, the evolutionary relationships within Macrobiotidae are only superficially determined as available genetic data cover only a small fraction of this vast group. Here, we present the first extensive molecular phylogeny of the family based on four molecular markers (18S rRNA, 28Sr RNA, ITS-2 and COI) associated with detailed morphological data for the majority of taxa. The phylogenetic analysis includes nearly two hundred sequences representing more than sixty species, including sixteen taxa that have never been sequenced and/or analysed phylogenetically before. Our results recovered a new monophyletic group, comprising *Macrobiotus spectabilis* Thulin, 1928 and *Macrobiotus grandis* Richters, 1911, for which we erect a new genus, *Sisubiotus* **gen. nov.**, to accommodate its evolutionary distinctiveness. The largest, so far, dataset for the family Macrobiotidae showed that the genus *Xerobiotus* is nested within the clade representing the genus *Macrobiotus* deeper than it was earlier assumed, therefore we propose to suppress *Xerobiotus* and transfer its species to *Macrobiotus*. Moreover, mapping key morphological traits onto macrobiotid phylogeny exposed complex evolution of phenotypes within the *Macrobiotus hufelandi* group, i.e. *Macrobiotus s.s.* Finally, our findings enabled a detailed revision and discussion on species compositions of the most ubiquitous tardigrade genera, species groups and species complexes, which resulted in changes of taxonomic statuses of a number of macrobiotid species. All this contributes to the reconstruction of the morphological evolution within Macrobiotidae.

1. Introduction

Tardigrades, are a phylum of microscopic, segmented and eight-legged invertebrates, closely related to arthropods and onychophorans which together form the super clade Panarthropoda (Campbell et al., 2011). They are found in various environments, from ocean depths to mountain peaks and from polar caps to tropical forests throughout the globe (Nelson et al., 2015). To date, the phylum comprises over 1300 species representing 142 genera and 30 families (Guidetti and Bertolani, 2005; Degma and Guidetti, 2007; Degma et al., 2009–2020).

The family Macrobiotidae Thulin, 1928 is one of the most species rich in the phylum and it comprises eutardigrades characterised by the absence of cephalic papillae, the presence of a compact epicuticular layer without pillar-like structures, double Y-shaped claws on each leg arranged symmetrically with respect to the median plane of the leg (configuration reported as: 2112), the presence of the ventral lamina (a strengthening bar on the ventral side of the buccal tube), and by laying ornamented eggs freely to the environment (Bertolani et al., 1996; Guidetti et al., 2000; Pilato and Binda, 2010; Marley et al., 2011). The family currently consist of nearly 300 species grouped within 14 genera, with 6 of them being originally classified as informal species groups/complexes within the super-diverse genus *Macrobiotus* Schultze, 1834 (*Biserovus* Guidetti and Pilato, 2003, *Calcarobiotus* Dastych, 1993, *Famelobiotus* Pilato et al., 2004, *Insuetifurca* Guidetti and Pilato, 2003, *Macrobiotus*, *Mesobiotus* Vecchi et al., 2016, *Minibiotus* Schuster, 1980, *Minilentus* Guidetti and Pilato, 2003, *Paramacrobiotus* Guidetti al., 2009, *Pseudodiphascon* Ramazzotti, 1965, *Pseudohexapodibius* Bertolani and Biserov, 1996, *Schusterius* Kaczmarek and Michalczyk, 2006, *Tenuibiotus*

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https://doi.org/10.1016/j.ympev.2020.106987
Received 14 May 2020; Received in revised form 3 October 2020; Accepted 7 October 2020
Available online 13 October 2020

Pilato and Lisi, 2010, *Xerobiotus* Bertolani and Biserov, 1996). The great majority of these taxa have been established based on morphology, with a clear monophyly confirmed by morphological and genetic data only for *Paramacrobiotus* and *Mesobiotus* (Guidetti et al., 2009; Vecchi et al., 2016). Moreover, after being split into numerous genera, *Macrobiotus* is still the largest and the most diverse taxon within the family and it remains polyphyletic (Bertolani et al., 2014). So far, the most studied species group within this genus has been the *Macrobiotus hufelandi* group (e.g. Bertolani and Rebecchi, 1993; Cesari et al., 2009; Bertolani et al., 2011a,b; Guidetti et al., 2013; Kaczmarek and Michalczyk, 2017; Stec et al., 2017a, 2018a, 2018b, 2018c; Coughlan and Stec, 2019; Kayastha et al., 2020). With records from all continents, the group has a cosmopolitan distribution (Kaczmarek and Michalczyk, 2017). Thirty years ago Biserov (1990a,b) proposed the formation of two subgenera within *Macrobiotus*: *Macrobiotus* and *Orthomacrobiotus*. The first comprised all then recognised species of the *hufelandi* group whereas the second grouped all remaining taxa assigned at the time to the genus *Macrobiotus*. However, this distinction was not commonly accepted and soon after Bertolani and Rebecchi (1993) discarded the idea of the two subgenera within *Macrobiotus*, questioning monophyly of the *Macrobiotus hufelandi* group. Notably, the position presented by the latter authors seemed to be actually more justified and real, especially in the light of recent studies which showed that the *Macrobiotus hufelandi* group is much more diverse than it was thought. Specifically, two distinct evolutionary lineages were found within this complex that were firstly thought to be congruent with divergent egg chorion morphology (Stec et al., 2018a), but subsequent discoveries falsified this hypothesis (Stec et al., 2018c; Coughlan and Stec, 2019).

The first molecular phylogeny of the family Macrobiotidae was constructed with COI sequences of eight species (Guidetti et al., 2005). Subsequent phylogenies, which were devoted to the whole phylum Tardigrada or the class Eutardigrada, were constructed using conservative markers such as 18S rRNA and 28S rRNA or their combination (Sands et al., 2008; Marley et al., 2011; Guil and Giribet, 2012; Bertolani et al., 2014; Guil et al., 2019). However, these phylogenies suffer from an underrepresentation of the less frequent and not easy to find taxa whereas ordinary, i.e. common species of the *Macrobiotus hufelandi* group and of the genera *Mesobiotus* and *Paramacrobiotus* are overrepresented. Moreover, many of the sequences used in these studies are not linked with morphological data and come from unidentified species what significantly limits evolutionary inference. Thus, taking into consideration the remarkable species diversity within Macrobiotidae, it seems that the current knowledge on the phylogenetic relationships within this taxon is biased and only superficially examined.

Therefore, in this study we present an extensive multilocus phylogeny of the family Macrobiotidae with 67 new sequences representing 16 taxa that have not been sequenced and/or have never been analysed phylogenetically. Moreover, we map key taxonomic traits onto the

Table 1
Information on moss samples with the species/populations of the family Macrobiotidae sequenced in the present study.

Sample/population code	Species	Locality	Coordinates and altitude	Collector
FI.066*	*Macrobiotus* cf. *pallarii*	Finland, Jyväskylä, Grannitti	62°13′24.60″N 25°46′20.40″E 84 m asl	Matteo Vecchi
ME.007	*Macrobiotus* cf. *pallarii*	Montenegro, Crkvine	42°47′57.54″N 19°27′18.47″E 1015 m asl	Aleksandra Rysiewska
PL.015	*Macrobiotus* cf. *pallarii*	Poland, Malinówka, Yew Reserve	49°42′09.00″N 21°55′53.00″E 382 m asl	Piotr Gąsiorek
US.057	*Macrobiotus* cf. *pallarii*	USA, Great Smoky Mountains National Park, Purchase Knob	35°35′7.84″N 83°4′26.47″W 1492 m asl	Nate Gross & Mackenzie McClay
AT.002	*Macrobiotus polonicus*	Austria, Purbach	47°54′56′'N 16°41′42′'E 130 m asl	Aneta Rumler
SK.003	*Macrobiotus polonicus*	Slovakia, Bratislava	48°8′54.70″N 17°7′2.39″E 145 m asl	Peter Degma
FI.068*	*Macrobiotus vladimiri*	Finland, Jyväskylä, Viitaniemi	62°15′15.10″N 25°43′36.10″E 94 m asl	Matteo Vecchi
FI.067*	*Sisubiotus spectabilis*	Finland, Jyväskylä, Survontie	62°13′45.80″N 25°44′39.50″E 95 m asl	Matteo Vecchi
NO.054	*Sisubiotus spectabilis*	Norway, vicinity of lake Avsjøen and E8 route	62°10′33.24″N 9°27′5.22″E 943 m asl	Daniel Stec & Witold Morek
ES.086	*Tenuibiotus* cf. *ciprianoi*	Spain, Aragón, Alerre, vicinity of Huesca	42°9′51.30″N 0°28′10.98″W 529 m asl	Piotr Gąsiorek & Witold Morek
KG.128	*Tenuibiotus danilovi*	Kyrgyzstan, Kum Dobo	42°13′22.62″N 75°27′17.82″E 2034 m asl	Bartłomiej Surmacz & Witold Morek
KG.140	*Tenuibiotus tenuiformis*	Kyrgyzstan, Toluk	41°55′8.70″N 73°37′49.44″E 1517 m asl	Bartłomiej Surmacz & Witold Morek
PL.360	*Xerobiotus* aff. *pseudohufelandi* sp. PL.360	Poland, Błędowska Desert	50°20′41.40″N 19°32′39.40″E 325 m asl	Daniel Stec & Krzysztof Miler
ZA.373	*Xerobiotus* aff. *pseudohufelandi* sp. ZA.373	South Africa, Cape of Good Hope, Western Cape	34°13′24.24″S 18°27′58.80″E 18 m asl	Bartłomiej Surmacz & Witold Morek

* Tardigrade Reproductive Evolution Group (University of Jyväskylä) sample codes: FI.066 = S14, FI.067 = S23, FI.068 = S15.

phylogeny and in result we reorganise macrobiotid systematics. On one hand, we demonstrate a novel clade comprising two species, *Macrobiotus spectabilis* Thulin, 1928 and *Macrobiotus grandis* Richters, 1911, for which we erect a new genus, *Sisubiotus* **gen. nov.** On the other hand, we supress the genus *Xerobiotus* since it is nested deep within the *Macrobiotus hufelandi* group. Our integrative analysis also exposes new species complexes and unveils complex morphological evolution in the *Macrobiotus hufelandi* group.

2. Material and methods

2.1. Samples and specimens

To reconstruct the phylogeny of the family Macrobiotidae, along with already published data, we analysed fourteen new populations representing eleven species isolated from moss samples collected from fourteen localities in different parts of the world (see Table 1 for details). In our study, by population we mean a group of conspecific individuals found in a single moss sample; furhermore, morphogroup is a non-monophyletic group of morphologically similar species; whereas species complexes are clades that cluster morphologically similar species. All samples were processed following a protocol described in detail in Stec et al. (2015).

2.2. Genotyping

Genomic DNA was extracted from individual animals following a Chelex® 100 resin (BioRad) extraction method by Casquet et al. (2012) with modifications described in detail in Stec et al. (2020a). Each specimen was mounted in water on a temporary microscope slide and examined under light microscope prior to DNA extraction. We sequenced four DNA fragments, three nuclear (18S rRNA, 28S rRNA, ITS2) and one mitochondrial (COI) from 2 to 4 individuals per each of the 14 newly analysed populations. All fragments were amplified and sequenced according to the protocols described in Stec et al. (2020a); primers with their original references are listed in Table 2. 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 NCBI GenBank (Wheeler et al., 2006).

2.3. Phylogenetic analysis

The phylogenetic analyses were conducted using concatenated 18S rRNA + 28S rRNA + ITS-2 + COI macrobiotid sequences with the families Murrayidae and Richtersiidae as outgroups. To reconstruct the phylogeny, we used sequences representing six different genera of Macrobiotoidea (Supplementary Materials, SM.01). Sequences were downloaded from GenBank (SM.01). We choose sequences from taxa identified to species level, including also some approximated identifications ("cf."). Only taxa with sequences for at least the 18S rRNA or the 28S rRNA locus were included in the phylogenetic reconstruction, with a few exceptions (for which only COI sequences were available and were included in the analysis) as sequences from the type or neotype populations are available and we considered them important for the completeness of our analysis: *Macrobiotus hufelandi* C.A.S. Schultze, 1834, *Macrobiotus macrocalix* Bertolani and Rebecchi, 1993 and *Macrobiotus vladimiri* Bertolani et al., 2011b. Finally, two very short 18S rRNA sequences classified as "*Minibiotus intermedius*" are present in GenBank (JX888505 and JX888504), however they were never officially published and they were not included in the analysis. Importantly, however, for the majority of taxa (more than 60%) the four markers were available, but none of the markers was present for all the taxa.

The 18S rRNA, 28S rRNA and ITS-2 sequences were aligned using MAFFT ver. 7 (Katoh et al., 2002; Katoh and Toh, 2008) with the G-INS-i method (thread = 4, threadtb = 5, threadit = 0, reorder, adjustdirection, anysymbol, maxiterate = 1000, retree 1, globalpair input) for ITS-2 and G-INS-i method allowing for unaligned regions (thread = 4, threadtb = 5, threadit = 0, reorder, adjustdirection, anysymbol, allowshift, unalignlevel = 0.1, maxiterate = 1000, retree = 1, globalpair input) for 18S rRNA and 28S rRNA. The COI sequences were aligned according to their aminoacid sequences (translated using the invertebrate mitochondrial code) with the MUSCLE algorithm (Edgar, 2004) in MEGA7 (Kumar et al., 2016) with default settings (all gap penalties = 0, max iterations = 8, clustering method = UPGMB, lambda = 24). Alignments were visually inspected and trimmed in MEGA7. Aligned sequences were concatenated with an in house R script wrote by MV. Model selection and phylogenetic reconstructions were done on the CIPRES Science Gateway (Miller et al., 2010). Model selection was performed for each alignment partition (8 in total: 18S rRNAa and b, 28S rRNAa and b, ITS-2 and three COI codons) with PartitionFinder2 (Lanfear et al., 2016), partitions and models selection process and results are present in Supplementary Material (SM.02). 18S and 28S alignments were divided in two partitions due to the presence in both of them of two regions differing in their occupancy matrix. BI phylogenetic reconstruction was done with MrBayes v3.2.6 (Ronquist et al., 2012) without BEAGLE. Four runs with one cold chain and three heated chains were run for 50 million generations with a burning of 5 million generations, sampling a tree every 1000 generations. Posterior distribution sanity was checked with the Tracer v1.7 (Rambaut et al., 2018) and with the R package RWTY (Warren et al., 2017). MrBayes input file with the input alignment is available as Supplementary Materials (SM.03). ML phylogenetic reconstruction was performed with RAxML-HPC Black Box 8.2.12 (Stamatakis, 2014) with 1000 bootstrap replicates and estimation of proportion of invariable sites (f = a, N = 1000, m = GTRCATI). The

Table 2
Primers with their original references used for amplification of the four DNA fragments sequenced in the study. Primer set LCO1490-JJ + HCO2198-JJ was used for COI amplification in six populations (FI.066, FI.067, NO.054, PL.015, PL.360, US.057); LCO1490 + HCO2198 was used in six populations (AT.002, ES.086, FI.068, KG.128, KG.140, SK.003); and LCO1490 + HCOoutout was used in two populations (ME.007, ZA.373). * – used only for *S. spectabilis* **comb. nov.** (FI.067).

DNA marker	Primer name	Primer direction	Primer sequence (5′-3′)	Primer source
18S rRNA	18S_Tar_Ff1	forward	AGGCGAAACCGCGAATGGCTC	Stec et al. (2017b)
	18S_Tar_Rr1	reverse	GCCGCAGGCTCCACTCCTGG	
28S rRNA	28S_Eutar_F	forward	ACCCGCTGAACTTAAGCATAT	Gąsiorek et al. (2018)
	28SR0990	reverse	CCTTGGTCCGTGTTTCAAGAC	Mironov et al. (2012)
28S rRNA*	28Sa	forward	GACCCGTCTTGAAACACGGA	Whiting et al. (1997)
	28Srd5b	reverse	CCACAGCGCCAGTTCTGCTTAC	Schwendinger and Giribet (2005)
ITS-2	ITS2_Eutar_Ff	forward	CGTAACGTGAATTGCAGGAC	Stec et al. (2018d)
	ITS2_Eutar_Rr	reverse	TCCTCCGCTTATTGATATGC	
COI	LCO1490-JJ	forward	CHACWAAYCATAAAGATATYGG	Astrin and Stüben (2008)
	HCO2198-JJ	reverse	AWACTTCVGGRTGVCCAAARAATCA	
	LCO1490	forward	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)
	HCO2198	reverse	TAAACTTCAGGGTGACCAAAAAATCA	
	HCOoutout	reverse	GTAAATATATGRTGDGCTC	Prendini et al. (2005)

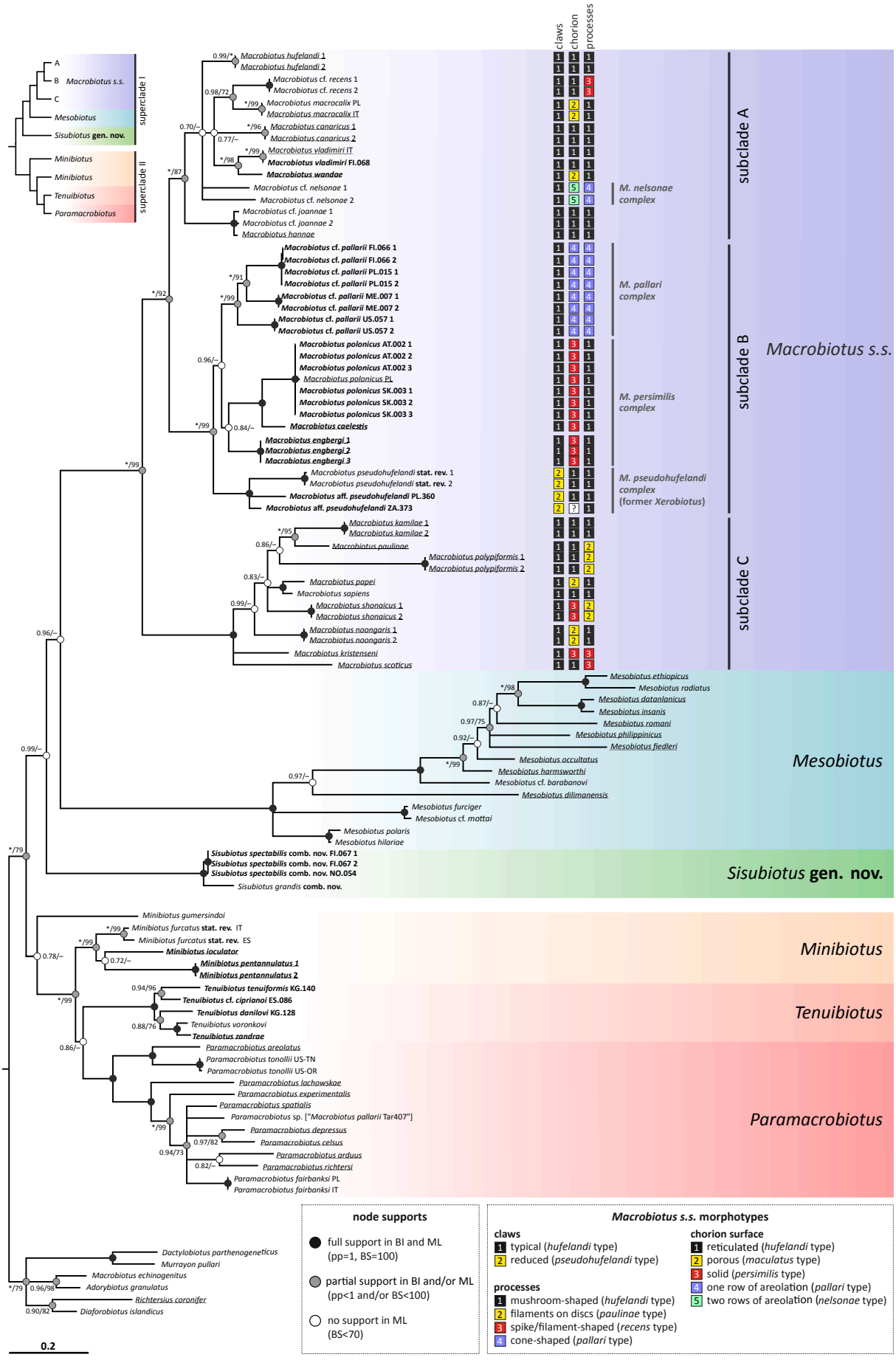

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Fig. 1. Phylogenetic reconstruction of the family Macrobiotidae with key morphological traits mapped onto the species of the *Macrobiotus hufelandi* group (=*Macrobiotus*). Topology from BI reconstruction, nodes below 0.70 posterior probability length were collapsed. Support values are indicated as BI posterior probability/ML bootstrap. Black circle and no value = full support in both analyses, i.e. 1 for BI or 100 for ML; grey circle = node supported in both analyses but not fully in at least one of them (* = full support, BI/ML values lower than 1/100 shown); white circle and an en dash = node supported only in BI (– = node with BS < 70% in the ML tree). Newly sequenced and/or newly analysed taxa/populations are bolded. Type, neotype and topotype sequences are underlined. Detailed legend for *Macrobiotus hufelandi* group morphotypes: **claws:** 1. *hufelandi* type (typical claws, as observed in *Mac. hufelandi*), 2. *pseudohufelandi* type (reduced claws with no lunules on legs I–III); **chorion surface:** 1. *hufelandi* type (reticulated), 2. *maculatus* type (porous), 3. *persimilis* type (solid), 4. *pallari* type (egg processes surrounded by one row of areolae), 5. *nelsonae* type (egg processes surrounded by two rows of areolae); **egg process shape:** 1. *hufelandi* type (mushroom-shaped), 2. *paulinae* type (mushroom-shaped with flexible filaments on terminal discs), 3. *recens* type (spike/filament-shaped), 4. *pallarii* type (conical with labyrinthine layer).

phylogenetic trees were visualised with FigTree v1.4.4 (Rambaut, 2007) and the image was edited with Inkscape 0.92.3 (Bah, 2011).

2.4. 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 differential interference contrasts (PCM and NCM, respectively; named collectively as light contrast microscopy, LCM), associated with an Olympus DP74 digital camera. In order to obtain clean and extended specimens for SEM, tardigrades were processed according to the protocol by Stec et al. (2015). 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 assembled in Corel Photo-Paint X6, ver. 16.4.1.1281. For structures that could not be satisfactorily focused in a single LCM 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.

2.5. Comparative material

Animals and eggs from the neotype series of *Mac. spectabilis* and *Mac. grandis* from the Maucci collection (Civic Museum of Natural History of

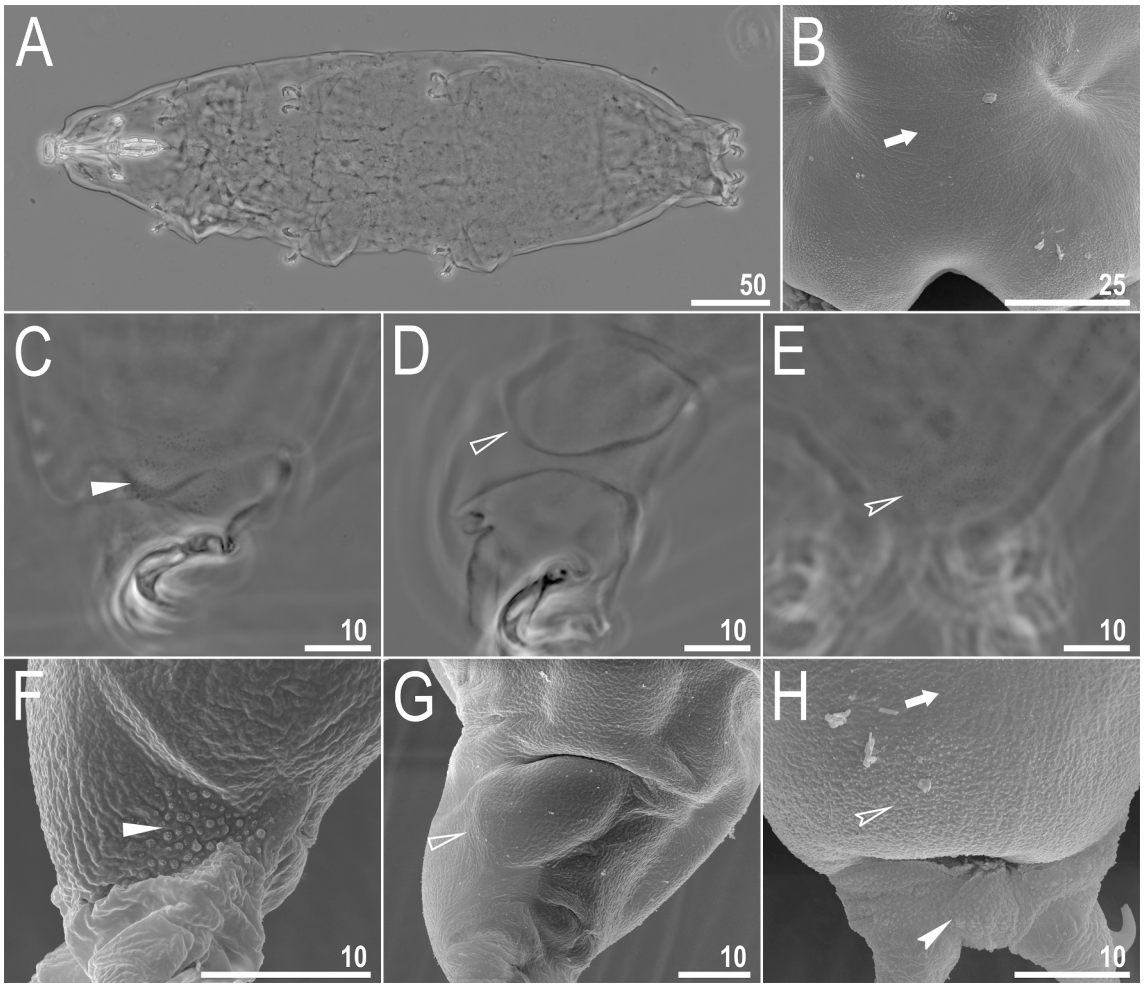

Fig. 2. *Sisubiotus spectabilis* **comb. nov.** from the Finnish population (FI.067) – habitus, body and leg cuticle morphology: **A** – adult specimen in dorso-ventral projection; **B** – granulation on the dorsal body cuticle visible in SEM; **C** – granulation on the external surface of leg II visible in LCM; **D** – internal surface of leg II with evident pulvinus visible in LCM; **E** – granulation on dorsal surface of leg IV visible in LCM; **F** – granulation on the external surface of leg II visible in SEM; **G** – internal surface of leg II with evident pulvinus visible in SEM; **H** – granulation on dorsal surface of leg IV visible in SEM. Arrows indicate fine body granulation visible only in SEM, filled flat arrowheads indicate granulation on the external surface of leg, empty flat arrowheads indicate a pulvinus-like cuticular bulge on the internal surface of leg, empty indented arrowheads indicate sparser and smaller part of granulation, filled indented arrowhead indicates denser and bigger part of granulation. Scale bar in μm.

Verona, Italy) were analysed and photographed under LCM, what allowed for a more detailed comparison with their original descriptions/redescriptions.

2.6. Morphometrics and 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 and Kaczmarek (2003) and Kaczmarek and Michalczyk (2017). Macroplacoid 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). Measurements of buccal tube widths, heights of claws and eggs follow Kaczmarek and Michalczyk (2017). Morphometric data were handled using the "Parachela" ver. 1.7 template available from the Tardigrada Register (Michalczyk and Kaczmarek, 2013) and are given in Supplementary Materials (SM.04). Tardigrade taxonomy follows Bertolani et al. (2014) with updates from Guidetti et al. (2016) and Vecchi et al. (2016).

3. Results

3.1. Phylogeny of Macrobiotidae

The BI and ML phylogenetic reconstructions yielded the same overall topology, but with lower bootstrap support values in the ML tree. Similarly to Bertolani et al. (2014), two major lineages within the family were recovered. In order to aid the description of phyletic relationships within the family Macrobiotidae, we term these lineages here as "superclade I" and "superclade II" (Fig. 1).

Superclade I comprises three clades: a clade with genera *Macrobiotus* (polyphyletic) and *Xerobiotus* (monophyletic but nested within *Macrobiotus*, thus designated here as invalid; see Discussion, Section 4), and further two clades, one corresponding to the genus *Mesobiotus* (monophyletic) and the other containing *Mac. grandis* and *Mac. spectabilis* (monophyletic, designated here as *Sisubiotus* **gen. nov.**; see below for the diagnosis of the new genus, Section 3.2.3). More specifically, *Macrobiotus* and *Mesobiotus* form a clade that is in a sister relationship with *Sisubiotus* **gen. nov.** Furthermore, the *Macrobiotus* + *Xerobiotus* clade is divided into three distinct evolutionary lineages (subclades A, B & C, respectively; Fig. 1). The first lineage (A) contains some species of the *Macrobiotus hufelandi* group as defined by Kaczmarek and Michalczyk (2017), including the type species for the genus (*Mac. hufelandi s.s.*), and species of the *Macrobiotus nelsonae* complex. Subclade B is morphologically the most diverse and comprises species of the *Macrobiotus persimilis* complex (falling under the broad definition of the *Macrobiotus hufelandi* group by Kaczmarek and Michalczyk (2017)), as well as species of the *Macrobiotus pallarii* complex, and species of the former genus *Xerobiotus*, now designated as the *Macrobiotus pseudohufelandi* complex. Finally, the lineage C comprises only some of the *Macrobiotus hufelandi* group species as defined by Kaczmarek and Michalczyk (2017).

Superclade II consists of *Minibiotus* (paraphyletic and with the nested *Macrobiotus furcatus* Ehrenberg, 1859, which hereby is retransferred to *Minibiotus*), *Tenuibiotus* (monophyletic), and *Paramacrobiotus* (monophyletic). More precisely, *Minibiotus gumersindoi* is a sister species to all remaining taxa constituting superclade II, but this node is not strongly supported suggesting a possible polytomy, whereas all other analysed *Minibiotus* spp. (including *Min. furcatus* (Ehrenberg, 1859) **stat. rev.**; see Discussion for a detailed justification, Section 4) form a clade that is in a

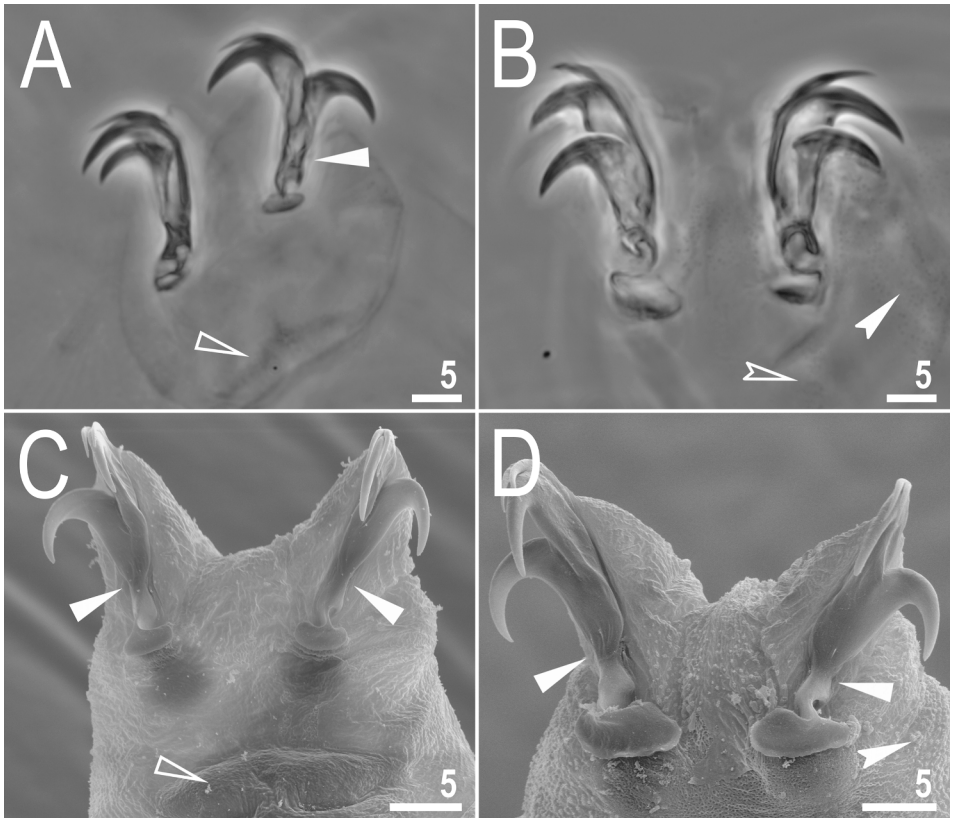

Fig. 3. *Sisubiotus spectabilis* **comb. nov.** from the Finnish population (FI.067) – claw morphology: **A–B** – claws I and IV seen in LCM; **C–D** – claws II and IV seen in SEM. Filled flat arrowheads indicate constriction in the claw common tract, empty flat arrowheads indicate double muscle attachments under claws, filled indented arrowheads indicate granulation on leg IV, the empty indented arrowhead indicates the faintly visible horseshoe-shaped structure. Scale bars in μm.

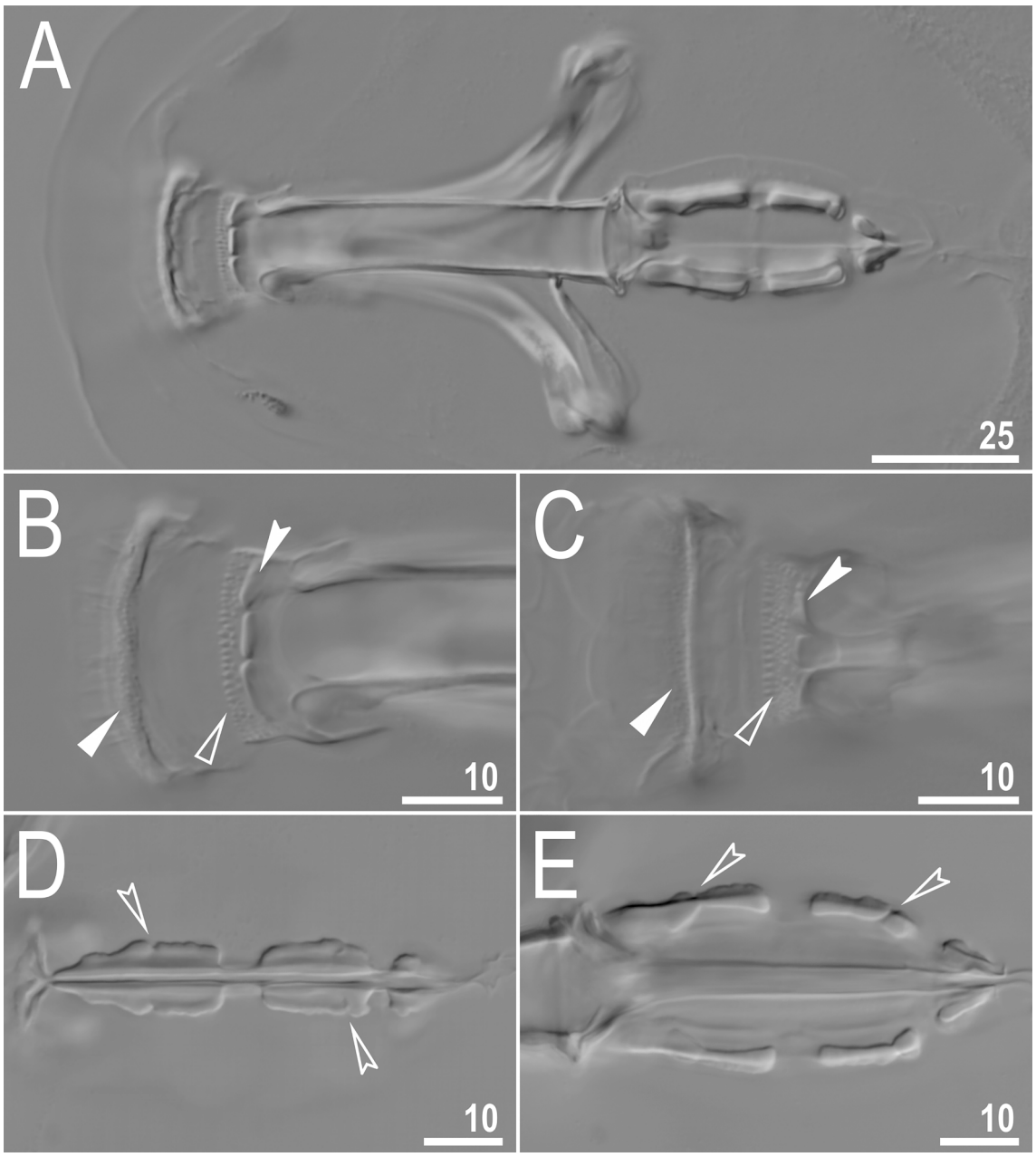

Fig. 4. *Sisubiotus spectabilis* **comb. nov.** from the Finnish population (FI.067) – buccal apparatus seen in LCM: **A** – an entire buccal apparatus; **B–C** – the oral cavity armature, dorsal and ventral teeth respectively; **D–E** – placoid morphology, dorsal and ventral placoids respectively. Filled 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 central and subterminal constrictions in the first and second macroplacoid, respectively. Scale bars in μm.

sister relationship to the clade formed by *Tenuibiotus* and *Paramacrobiotus*.

To sum up, the following genera were found to be monophyletic: *Mesobiotus*, *Sisubiotus* **gen. nov.**, *Tenuibiotus* and *Paramacrobiotus*. *Minibiotus* is paraphyletic with respect to the *Tenuibiotus* + *Paramacrobiotus* clade. Moreover, the most specious genus within the family, *Macrobiotus*, is polyphyletic with *Macrobiotus echinogenitus* (MH079513, MH079460) being akin to *Adorybiotus granulatus* as shown in Guil et al. (2019). Finally, GenBank sequences from the isolate Tar407 (FJ435741, FJ435756, FJ435807) represent an unidentified *Paramacrobiotus* species which was misidentified by Guil and Giribet (2012) as *Macrobiotus pallarii* Maucci, 1954.

3.2. Description of the new genus

3.2.1. Systematic and taxonomic account

Phylum: Tardigrada Doyère, 1840
Class: Eutardigrada Richters, 1926
Order: Parachela Schuster et al., 1980 (restored by Morek et al., 2020)
Superfamily: Macrobiotoidea Thulin, 1928
Family: Macrobiotidae Thulin, 1928
Genus: *Sisubiotus* **gen. nov.** Stec, Vecchi, Calhim and Michalczyk (Figs. 1–7)

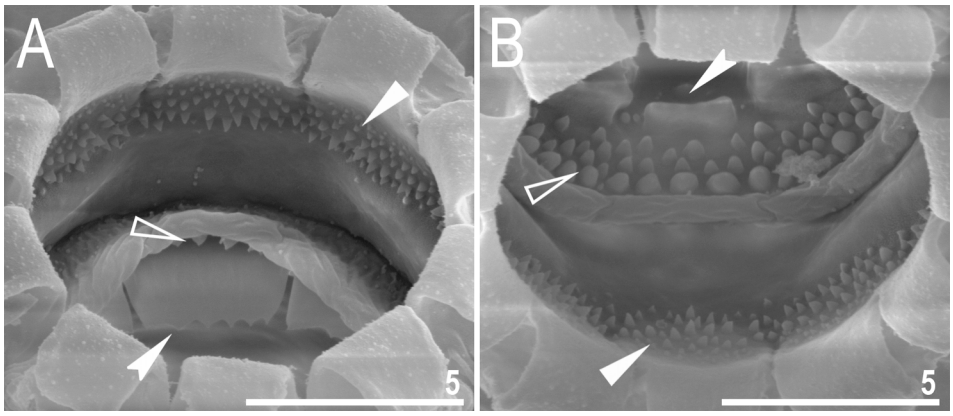

Fig. 5. *Sisubiotus spectabilis* **comb. nov.** from the Finnish population (FI.067) **–** the oral cavity armature seen in SEM: **A–B** – the oral cavity armature of a single specimen seen in SEM from different angles showing dorsal and ventral portion, respectively. Filled flat arrowheads indicate the first band of teeth, empty flat arrowheads indicate the second band of teeth, filled indented arrowheads indicate the third band of teeth. Scale bars in μm.

3.2.2. Etymology

The union of *Sisu*, a Finnish concept described as stoic determination, tenacity of purpose, grit, bravery, resilience, and hardiness that seems to fit well the resistance capabilities of tardigrades, and *biotus*, a common generic suffix in Macrobiotoidea.

3.2.3. Diagnosis

Large and whitish Macrobiotidae with: (*i*) poreless cuticle, (*ii*) mouth opening surrounded by ten peribuccal lamellae, (*iii*) buccal apparatus of the *Macrobiotus* type, with a wide rigid buccal tube and a well-developed oral cavity armature (all three bands of teeth clearly visible in LCM,

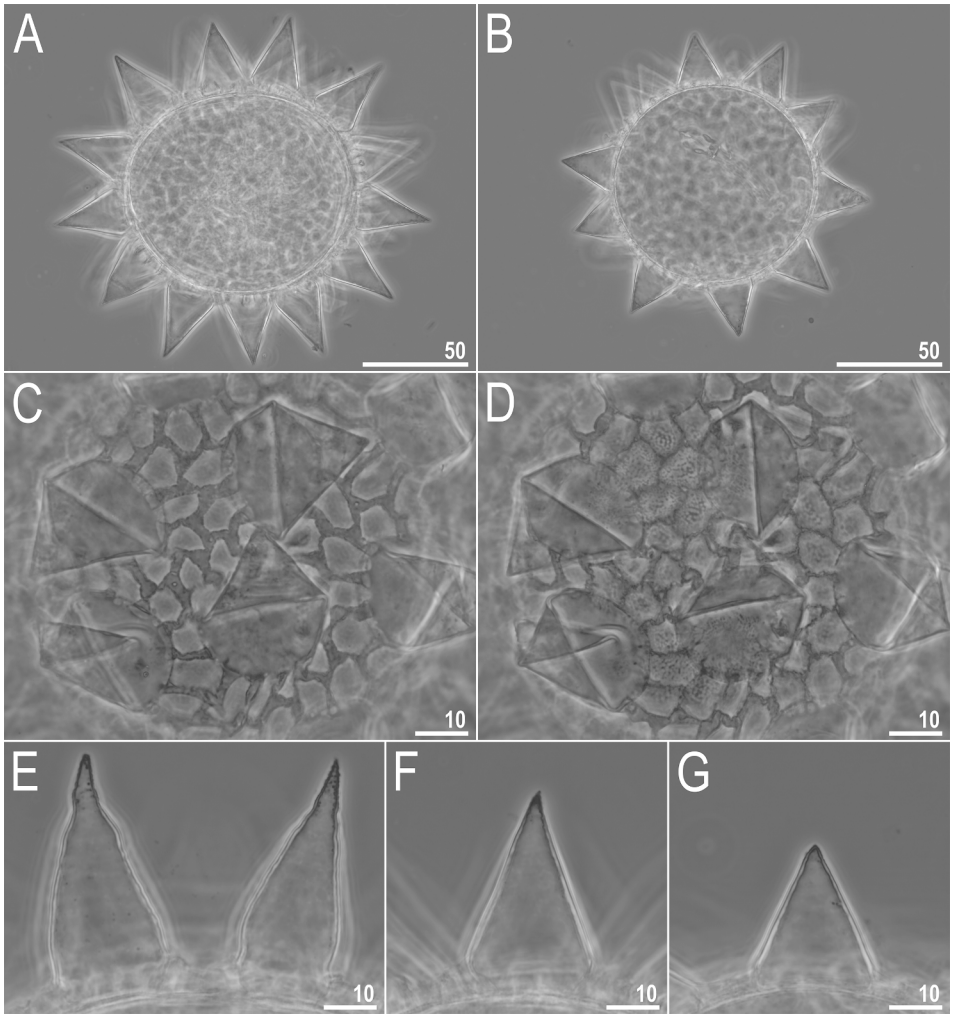

Fig. 6. *Sisubiotus spectabilis* **comb. nov.** from the Finnish population (FI.067) – eggs seen in LCM: **A–B** – midsections of two different eggs under ×400 magnification; **C–D** – surface under ×1000 magnification for the same eggs on two different focus levels; **E–G** – midsections of four different egg processes. Scale bars in μm.

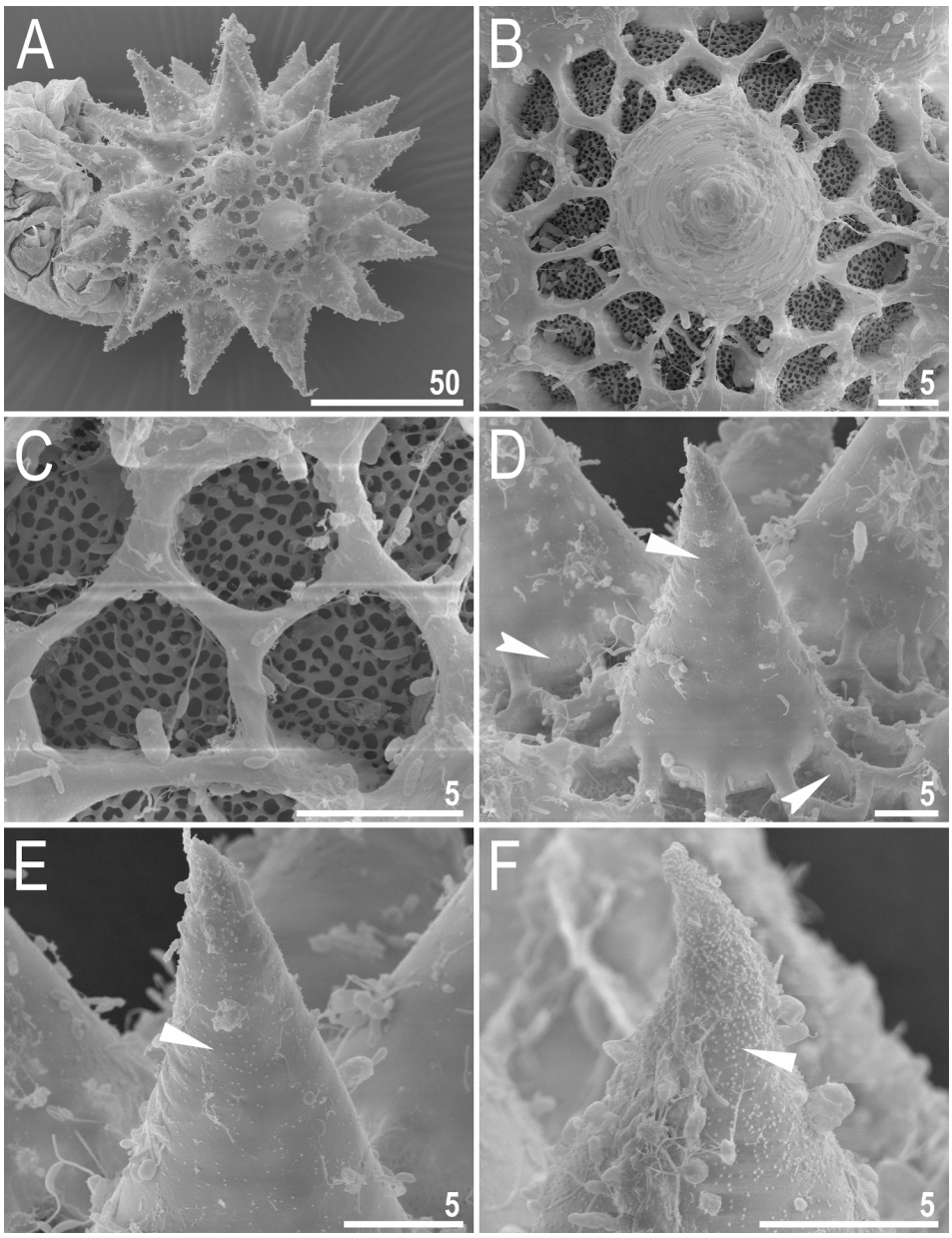

Fig. 7. *Sisubiotus spectabilis* **comb. nov.** from the Finnish population (FI.067) – eggs seen in SEM: **A** – entire view of the egg; **B–C** – details of the egg surface between processes and areolation; **D** – egg process; **E–F** – top part of the processes covered with microgranulation. Filled flat arrowheads indicate the granulation on processes apices, filled indented arrowheads indicate microgranulation on the internal walls of areoles. Scale bars in μm.

anterior teeth of the second band longitudinally elongated), (*iv*) two macroplacoids and a large microplacoid positioned close to them, (*v*) Y-shaped claws of the modified *hufelandi* type with lunules on each leg, (*vi*) ornamented eggs, laid freely, with areolation and conical processes without the labyrinthine layer that in many other macrobiotids is visible as reticulation in LCM.

3.2.4. Genus composition

Sisubiotus spectabilis (Thulin, 1928) **comb. nov.** (type species)

Sisubiotus grandis (Richters, 1911) **comb. nov.**

Sisubiotus wuyishanensis (Zhang & Sun, 2014) **comb. nov.** (*species inquirenda*, please see the Discussion for details, Section 4.1.)

Although *S. grandis* **comb. nov.** was described earlier than *S. spectabilis* **comb. nov.**, the latter taxon is characterised in much more detail in this study, thus in order to secure the stability of taxonomy within the new genus, we designate *S. spectabilis* **comb. nov.** as the type species for *Sisubiotus* **gen. nov.**

3.2.5. Differential diagnosis

Sisubiotus **gen. nov.**, by the combination of morphological characters of animals and eggs is unique in the family Macrobiotidae, and it differs specifically from:

- *Biserovus* Guidetti and Pilato, 2003 by: the absence of the flexible pharyngeal tube, the number of macroplacoids in the pharynx (two in the new genus *vs* three in *Biserovus*) and the presence of lunules under claws (lunules absent in *Biserovus*).
- *Calcarobiotus* Dastych, 1993 by: the number of macroplacoids in the pharynx (two in the new genus *vs* three in *Calcarobiotus*) and a different claw type (the basal section of all claws without spurs, subdivided into a thin flexible stem and a distal section which is poorly sclerified and distally delimited by a septum, primary and secondary branches are rigidly joined to each other along a long common tract in *Sisubiotus* **gen. nov.** *vs* the basal section of each claw with or without basal spurs, subdivided into a thin flexible stem and

a wide distal section in the shape of an upside-down triangle which is distally delimited by a septum, primary and secondary branches are similar in shape and size in *Calcarobiotus*).

- *Famelobiotus* Pilato et al., 2004 by: the number of macroplacoids in the pharynx (two in the new genus *vs* three in *Famelobiotus*) and the absence of the double third band of teeth in the oral cavity armature.
- *Insuetifurca* Guidetti and Pilato, 2003 by: the number of macroplacoids in the pharynx (two in the new genus *vs* three in *Insuetifurca*) and the absence of the flexible pharyngeal tube.
- *Macrobiotus* Schultze, 1834 by: the absence of pores in the cuticle, the longitudinally elongated teeth in the anterior row of the second band of teeth of the oral cavity (when present, teeth only round in *Macrobiotus*; except for *Mac. andinus* Maucci, 1988, which could represent a separate lineage), and the lack of the labyrinthine layer in conical egg processes (when present, conical processes with clearly visible reticulation in LCM, at least in the lower part of the process, in *Macrobiotus*).
- *Mesobiotus* Vecchi et al., 2016 by: the number of macroplacoids in the pharynx (two in the new genus *vs* three in *Mesobiotus*) and the lack of the labyrinthine layer in conical egg processes (when present, conical processes with clearly visible reticulation in LCM in *Mesobiotus*).
- *Minibiotus* Schuster, 1980 by: the presence of peribuccal lamellae (peribuccal papulae in *Minibiotus*; but see also Stec et al. (2020a)), the number of macroplacoids in the pharynx (two in the new genus *vs* usually three in *Minibiotus*), adult body length (up to 900 μm in the new genus *vs* no more than 400 μm in *Minibiotus*). *Remarks*. *Minibiotus* comprises divergent morphotypes, e.g. species with two and three macroplacoids in the pharynx, species with smooth and porous cuticle, and species in which these characters are intermixed. This strongly suggests that *Minibiotus* is polyphyletic (see the Discussion below).
- *Minilentus* Guidetti and Pilato, 2003 by: the number of macroplacoids in the pharynx (two in the new genus *vs* three in *Minilentus*), the presence of peribuccal lamellae (lamellae absent in *Minilentus*), and the absence of the flexible pharyngeal tube.
- *Paramacrobiotus* Guidetti et al., 2009 by: the number of macroplacoids in the pharynx (two in the new genus *vs* three in *Paramacrobiotus*), the distance between the third macroplacoid and the microplacoid (the distance shorter than the microplacoid *vs* longer than the microplacoid in the *P. richtersi* complex or the microplacoid lacking in the *P. areolatus* morphogroup), and the lack of the labyrinthine layer in egg processes (processes with clearly visible reticulation in *Paramacrobiotus*, excluding *Paramacrobiotus csotiensis* (Iharos, 1966a, 1966b)).
- *Pseudohexapodibius* Bertolani and Biserov, 1996 by: the claw morphology (Y-shaped claws of the *hufelandi* type with lunules in the new genus *vs* claws I–III strongly reduced, claws on legs IV rudimental or absent, lunules absent in *Pseudohexapodibius*).
- *Schusterius* Kaczmarek and Michalczyk, 2006 by: the number of macroplacoids in the pharynx (two in the new genus *vs* three in *Schusterius*) and the morphology of accessory points on the primary branches of claws (typical, short in the new genus *vs* extremely elongated and connected to primary branches by a flexible light-refracting portion in *Schusterius*).
- *Tenuibiotus* Pilato and Lisi, 2010 by: the claw morphology (primary and secondary branches joined over a shorter distance and the remaining free portion of the secondary branch forming an acute angle with the primary branch in the new genus *vs* primary and secondary branches joined over a long distance and the remaining free portion of the secondary branch is clearly shorter than the primary branch, the branches form an almost right angle in *Tenuibiotus*). *Remarks*. *Tenuibiotus* most likely comprises more than one genus, as it contains species with two and three macroplacoids as well as species with and without pores in the cuticle (for more details please see the Discussion below, Section 4.1.).

4. Discussion

4.1. The phylogeny and systematics of Macrobiotidae

The family Macrobiotidae is characterised by symmetrical claws and its monophyly has been consistently demonstrated by earlier phylogenetic studies (Marley et al., 2011; Bertolani et al., 2014). However, the relationships between the macrobiotid genera and their composition are only partially resolved mainly because: *(i)* the available Macrobiotoidea phylogenetic trees are taxonomically biased, i.e. they largely comprise the most common species and lack many of the rare taxa, some of which may be crucial for the understanding macrobiotid evolution; *(ii)* even if sequences are available, they do not always represent homologous fragments of a given marker (this concerns especially the 18S and 28S rRNA); and *(iii)* for many species only a single marker is known, which results in missing data in the alignments, impeding the resolution of some phylogenetic relationships, especially with Maximum Likelihood phylogenetic methods that require relatively large amounts of data to obtain high bootstrap confidence values on short internodes (Alfaro et al., 2003). Specifically, there are no molecular data for eight out of the currently fifteen recognised macrobiotid genera: *Biserovus*, *Calcarobiotus*, *Famelobiotus*, *Insuetifurca*, *Minilentus*, *Pseudodiphascon*, *Pseudohexapodibius*, and *Schusterius*. These genera are extremely rare and, except for *Calcarobiotus* and *Insuetifurca*, they are all monotypic. The remaining six genera, for which DNA sequences are available (*Macrobiotus*, *Mesobiotus*, *Minibiotus*, *Paramacrobiotus*, *Tenuibiotus*, and *Xerobiotus*), have been used in previous tardigrade phylogenetic studies (e.g. Guil and Giribet, 2012; Bertolani et al., 2014; Stec et al., 2018c; Guil et al., 2019). Notably, however, these phylogenies were based on one or two conservative ribosomal markers and on species for which DNA sequences often were not associated with detailed morphological data, making their identifications uncertain (species identified to the genus/complex level or with the confer "cf." species status) and frequently also erroneous (please see Morek et al. 2019; Grobys et al. 2020; Stec et al. 2020b who found identification errors in some earlier studies). In contrast, our study provides, for the very first time, an extensive multilocus phylogeny, based on four molecular markers for species with much better documented phenotypes than ever before. This allowed for more precise conclusions and led to the discovery of new evolutionary lineages and a new genus, but – at the same time – also led to grouping some other taxa within the family. Moreover, our results underline problems with species composition of some genera analysed in this study, which we discuss below (the order follows Fig. 1 from top to bottom).

Macrobiotus, the first described tardigrade genus, is the most speciose and diverse in the family even though many of its members have been stripped away to create new genera (see Introduction for details). The genus currently comprises 117 species, but only for ca. 6% of them have any DNA markers been sequenced. Moreover, the majority of *Macrobiotus* sequences (together with the suppressed *Xerobiotus*) form a monophyletic clade. Only *Mac. furcatus* and *Mac. echinogenitus* are not directly related with all the other analysed *Macrobiotus* species. Given that we have moved *Mac. furcatus* to the genus *Minibiotus* in this study, before discussing the *Macrobiotus s.s.* clade, only *Mac. echinogenitus* needs to be addressed. The DNA sequences of an individual from Greenland attributed to this species were produced by Guil et al. (2019) and their as well as our phylogenetic analyses point to *Adorybiotus* (Richtersiidae) as its closest kin. Three possible explanations for the results of Guil et al. (2019) can be given: (i) the sequences are from a legit *Mac. echinogenitus* individual and this species should be ascribed to *Adorybiotus* or (ii) to a new genus; (iii) this is a misidentified *Macrobiotus crenulatus* Richters, 1904, which has been moved to the recently erected genus *Crenubiotus* Lisi et al., 2020. The third possibility seems to be most likely given the long history of confusion between *Mac. echinogenitus* and *Mac. crenulatus* (Ramazzotti and Maucci, 1983; Binda, 1988). Nevertheless, in all cases, an integrative redescription of *Mac. echinogenitus*

and/or *Crenubiotus* DNA sequences are needed to solve this problem.

After suppressing the genus *Xerobiotus* and transferring its species to *Macrobiotus*, moving *Macrobiotus furcatus* back to *Minibiotus*, and assuming that GenBank sequences labelled as "*Mac. echinogenitus*" represent a species of the family Richtersiidae, all other *Macrobiotus* species analysed in our study form a monophyletic lineage, named here *Macrobiotus s.s.*, which is further divided into three subclades A, B and C (Fig. 1). All species in this lineage conform to the definition of the *Macrobiotus hufelandi* group, proposed by Bertolani and Rebecchi (1993) and further refined by Guidetti et al. (2013), that encompasses species with animals exhibiting porous cuticle, up to three bands of teeth in the oral cavity, and two macroplacoids and a microplacoid in the muscle pharynx. Kaczmarek and Michalczyk (2017) attempted to narrow the definition to make it more practical taxonomically by adding three more criteria: (i) Y-shaped claws with lunules on all the legs, (ii) ornamented eggs with single-walled processes that are most often terminated with a distinct disc, (iii) and egg surface never covered with areolation. The first criterion was introduced to exclude *Xerobiotus* spp. and the two remaining criteria excluded species with conical and reticulated egg processes: *Mac. pallari* and *Macrobiotus nelsonae* Guidetti, 1998. At the time, the first of these two species was considered to be related to *Paramacrobiotus* as it clustered with other species of that genus (Guil and Giribet, 2012; Bertolani et al., 2014; Guil et al., 2019). However, now, when more *Paramacrobiotus* and *Mac.* cf. *pallari* species have been sequenced, the single specimen identified by Guil and Giribet (2012) as "*Mac. pallari*" clusters with *Paramacrobiotus* spp., whereas the several correctly identified *Mac.* aff. *pallari* species are nested deeply within *Macrobiotus s.s.* (Fig. 1). Thus, now, when *Mac.* aff. *pallari* species have been sequenced, it became apparent that Guil and Giribet (2012) misidentified their specimen and the flawed sequences were repeatedly used in later studies (i.e. Bertolani et al., 2014; Guil et al., 2019), what explicitly shows that not all GenBank sequences can be trusted and that misidentifications may have long-term detrimental effects and impede progress in taxonomy and systematics. The second species, *Mac. nelsonae*, was considered a likely sample labelling error because egg morphology significantly departs from the classical *Mac. hufelandi* morphotype. Nevertheless, Kaczmarek and Michalczyk (2017) stated that their narrowed diagnosis of the *Mac. hufelandi* group should be considered as a "working definition" to aid species identification. However, our phylogenetic analysis, by demonstrating the presence of a

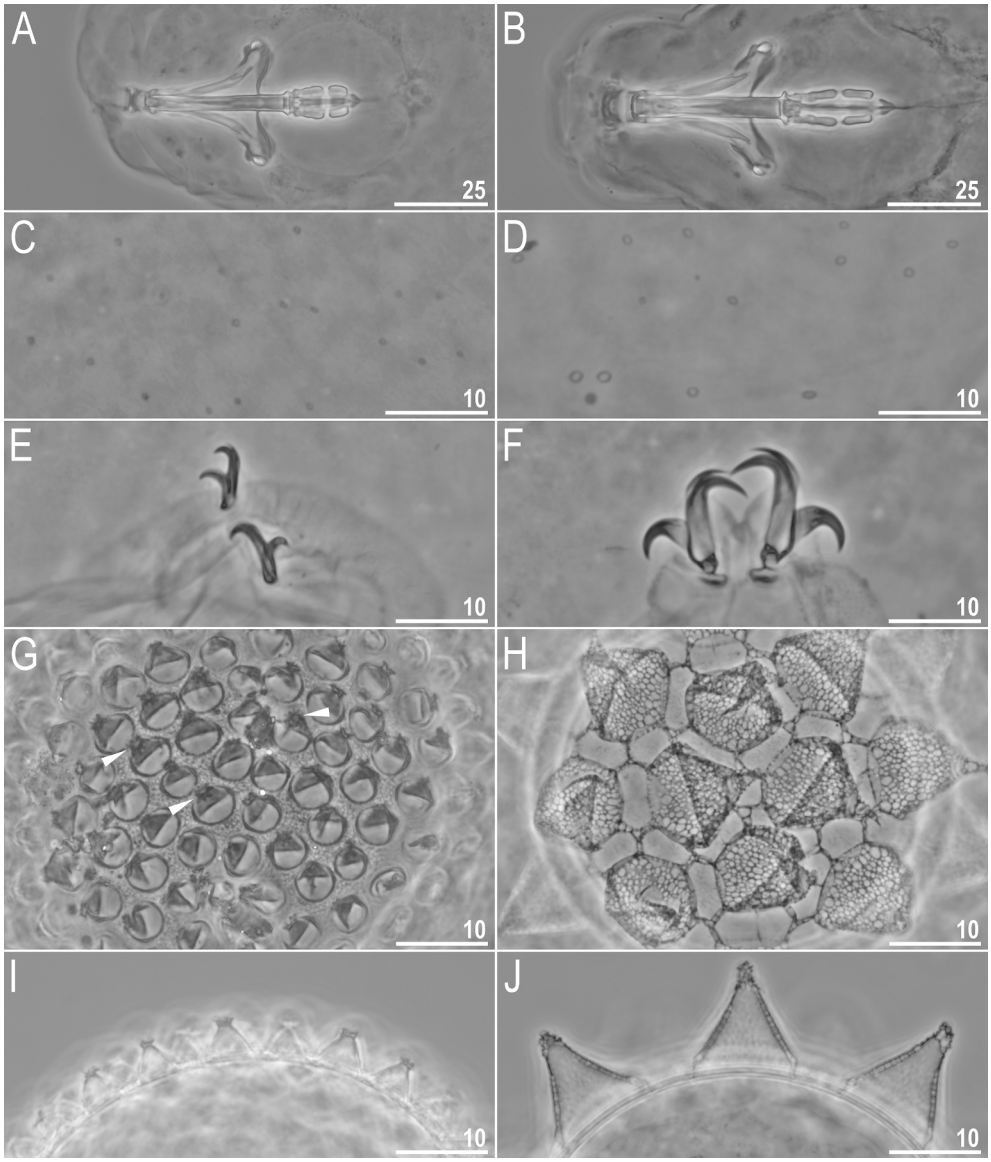

Fig. 8. Comparative morphological figure of *Xerobiotus* sp. from Poland (PL.360, left column) and *Macrobiotus* cf. *pallarii* from Montenegro (ME.007, right column): **A–B** – buccal apparatus; **C–D** – cuticular pores; **E–F** – claws on leg III; **G–H** – egg surface under ×1000 magnification; **I–J** – egg midsection under ×1000 magnification. Filled flat arrowheads indicate terminal discs with indented margins on the egg processes. Scale bars in μm.

potential *Mac. nelsonae* complex in subclade A and the presence of the *Mac. pallarii* and *Mac. pseudohufelandi* complexes in subclade B, leaves no doubt that the definition by Kaczmarek and Michalczyk (2017) encompasses a polyphyletic group of species. Thus, in the current dataset the genus *Macrobiotus s.s.* is equivalent to the *Mac. hufelandi* morphogroup as defined by Guidetti et al. (2013), with the inclusion of species of the supressed *Xerobiotus*.

The three subclades within *Macrobiotus s.s.*, exhibiting an [(A + B) + C] topology, are clearly delineated in our analysis (Fig. 1). However, with hindsight, they can also be identified in earlier studies, Bertolani et al. (2014) and Stec et al. (2018c), although with a different topology, [(A + C) + B], and a weaker support. In Bertolani et al. (2014), the subclades were not noticed whereas in Stec et al. (2018c) clade B was considered an artefact because it contained *Macrobiotus* and *Xerobiotus* species. Thus, the present study is the first to acknowledge the three lineages within *Macrobiotus s.s.* Noting this clear phylogenetic division of the *Macrobiotus s.s.* clade, we attempted to find morphological synapomorphies for the three subclades to investigate the possibility of designating them as separate taxonomic units such as subgenera or genera of their own. However, we identified no traits or their combinations that would be exclusive to each subclade. For example, reduced (*pseudohufelandi* type) claws are present only in some species of subclade B, eggs with a reticulated chorion (*hufelandi* type) occur in all subclades, eggs with smooth chorion (*persimilis* type) are present in subclades B and C, cone-shaped egg processes (*pallari* type) are found in subclades A and B, and single-walled mushroom-shaped egg processes (*hufelandi* type) occur in all subclades, etc. There are, however, four complexes that cluster closely related species exhibiting unique and uniform morphology. Even though these complexes do not align with the three subclades, delineating them may be taxonomically useful: the *Macrobiotus nelsonae* complex (characterised mainly by two rows of areolae between egg processes), *Macrobiotus pallari* complex (one row of areolae between egg processes), *Macrobiotus persimilis* complex (smooth egg chorion with mushroom-shaped processes, noted earlier by Bertolani et al. (2012; 2018) and Kaczmarek and Michalczyk (2009)), and *Macrobiotus pseudohufelandi* complex (reduced claws); see Table 5 for more detailed diagnoses and species compositions of each complex. The *Mac. nelsonae* complex is nested within subclade A, whereas the remaining three complexes constitute the entire subclade B. For the time being, we propose to gather, for purely taxonomic purposes, all other *Macrobiotus hufelandi* group species listed by Kaczmarek and Michalczyk (2017) in a polyphyletic *Macrobiotus hufelandi* morphogroup as defined in Table 5. Nevertheless, it should be noted that there are other morphologically unique species or groups of species within the genus *Macrobiotus*, such as the *Macrobiotus polyopus* group (see Pilato 2006) or *Macrobiotus ariekammensis* group (see Tumanov 2005), for which there are no molecular data and their phylogenetic position has not yet been determined. If they turn out to be monophyletic and not nested within the *Macrobiotus s.s.*, they are likely to become genera in their own right, similarly to *Paramacrobiotus*, *Mesobiotus* or *Sisubiotus* **gen. nov.** However, if they form clades nested within *Macrobiotus*, they could be delineated as species complexes similarly to *Mac. nelsonae*, *Mac. pallarii*, *Mac. persimilis*, and *Mac. pseudohufelandi* complexes identified here (Table 6). Also, it may be also worth investigating whether such species complexes with similar morphology also exhibit similar ecological characteristics, such as diet, microhabitat type or geographic distribution.

Xerobiotus was erected by Bertolani and Biserov (1996) using exclusively phenotypic characters. Specifically, the genus was established to accommodate two *Macrobiotus* species, *Mac. pseudohufelandi* Iharos, 1966 and *Mac. xerophilus* Dastych, 1978, that exhibit extremely reduced claws and lunules only on the hind legs (in all other *Macrobiotus* species, claws are not reduced and they all are equipped with lunules). Up to date, only three *Xerobiotus* species, all with a limited number of records, have been described. However, in the light of current standards in tardigrade taxonomy, their descriptions can be considered as vague due to the insufficient documentation of morphological details that are vital for the identification of phenotypically similar species. Moreover, only for a single species, *X. pseudohufelandi*, are DNA sequences available (18S rRNA and COI from Guidetti et al., 2005; Bertolani et al., 2014), thus it was the only of the described species that could be used in our study. In earlier phylogenetic studies of the family Macrobiotidae (Bertolani et al., 2014; Stec et al., 2018c), *X. pseudohufelandi* clustered together with *Mac. polonicus* Pilato et al., 2003 in a clade that was sister to the remaining clades of the *Macrobiotus hufelandi* group, thus there was an expectation that with sequences for more species, *Xerobiotus* will become a clade that is sister to all species of the *Mac. hufelandi* group (Stec et al., 2018c). In the present study, we provided new molecular data by sequencing four molecular markers for further two yet undescribed *Xerobiotus* species, one from Europe (Poland) and the other from Africa (RSA) (Table 1). Instead of separating *Xerobiotus* from *Macrobiotus*, our analysis showed that all sequenced *Xerobiotus* species form a well-supported clade nested deeply within clade B (Fig. 1), rendering the hypothesis that *Xerobiotus* constitutes an independent phyletic line unlikely (hence the genus is supressed and its species are grouped in the *Macrobiotus pseudohufelandi* complex; see Table 5). In fact, apart from the claw morphology, *Xerobiotus* is morphologically indistinguishable from the *Macrobiotus hufelandi* group as both taxa share the same buccal apparatus and spermatozoon morphology (Dastych & Alberti 1990; Guidi and Rebecchi, 1996; Rebecchi et al., 2000, 2011; Guidetti et al., 2005; Bertolani et al., 2014). Although the presence of cuticular pores, one of the key traits defining the *Mac. hufelandi* group, has not been verified by SEM in all *Mac. pseudohufelandi* complex species, it seems very likely that they are present but their diameter is below the LCM resolution (see Kaczmarek and Michalczyk 2017 for a detailed discussion on this). Moreover, egg shell ornamentation with mushroom-shaped processes in *Mac. pseudohufelandi* (Iharos, 1966a, 1966b) **stat. rev.** and in the unidentified species from Poland, *Mac.* aff. *pseudohufelandi* sp. PL.360 (Fig. 8), is typical for the majority of species of the *hufelandi* group (eggs are not known for *Mac. euxinus* (Pilato et al., 2011) **comb. nov.** and for the South African species analysed herein; *Mac. xerophilus* (Dastych, 1978) **stat. rev.** exhibits a unique type of egg ornamentation, but in recent years various new egg morphotypes were found in some species of the *hufelandi* group, e.g. in *Mac. kristenseni* Guidetti et al., 2013 or *Mac. scoticus* Stec et al., 2017a, thus a deviation from the typical process shape does not exclude a species from the *Mac. hufelandi* group; see Table 5 for examples). Thus, it seems that reduced claws in the *Mac. pseudohufelandi* complex are a relatively recent adaptation to dwelling in dense media such as soil that evolved in a single lineage within the *Mac. hufelandi* group that otherwise inhabits mostly much less dense mosses. Therefore, one way to preserve the genus would be to split the *Mac. hufelandi* group into three genera that correspond with clades A–C in Fig. 1. This, however, is not possible as currently there are no recognisable apomorphies that would allow for the erection of such genera (see above for details). Another solution to keep *Xerobiotus*, would be to erect new genera for the two other species complexes within subclade B identified in this paper, i.e. the *Mac. pallari* and the *Mac. persimilis* complex. However, under such scenario, the remaining species of the currently monophyletic genus *Macrobiotus* with a well-defined synapomorphy (porous cuticle combined with the buccal apparatus of the *Mac. hufelandi* type) would become polyphyletic with no foreseeable chances for the delineation of genera to accommodate species in subclades A and C (the same morphotypes are present within these subclades; see Fig. 1). Moreover, given that the majority of *Macrobiotus* species have not yet been sequenced, the apomorphies may be broken if species representing divergent phenotypes are found to be nested within the currently recognised complexes or species exhibiting similar phenotypes cluster in different subclades. Therefore, taking into consideration the above, the most parsimonious solution is to supress the genus *Xerobiotus* and retransfer all its species to *Macrobiotus* with new combinations as members of the *Macrobiotus pseudohufelandi* complex (please see the Systematic account, Section 5.1. and Table 5 for details).

Mesobiotus was erected by an integrative analysis of two former species complexes in the genus *Macrobiotus*, the *harmsworthi* and the *furciger* groups. Vecchi et al. (2016) demonstrated that these groups are morphologically different from other macrobiotid genera and form a monophyletic clade. The monophyly of *Mesobiotus* was positively verified by Guil et al. (2019) and the present study. Similarly to the genus *Paramacrobiotus*, species representing the two species groups within *Mesobiotus* are intermixed, thus currently no subgeneric ranks can be established to accommodate them (Kaczmarek et al. 2018).

The new genus ***Sisubiotus***, erected in this study, comprises currently only three species. Two of them, *S. spectabilis* **comb. nov.** and *S. grandis* **comb. nov.** have been considered for many years as *species inquirenda* (Dastych, 1973). However, the study by Maucci and Pilato (1974) showed that these two species are valid and they can be distinguished by the position of eyes (anterior in *S. spectabilis* **comb. nov.** *vs* posterior in *S. grandis* **comb. nov.**), the morphology of the second band of teeth in the oral cavity (additional rows of small teeth in *S. spectabilis vs* second band almost absent or composed by a few teeth in *S. grandis* **comb. nov.**), the size of macroplacoids (longer in *S. spectabilis* **comb. nov.**) and details of the egg shell (reticulated areola surface and smooth processes in *S. spectabilis* **comb. nov.** *vs* areola surface without reticulation and processes with small opaque areas in *S. grandis* **comb. nov.**). Although Dastych (1973) redescribed *S. spectabilis* **comb. nov.** based on a Polish population, the neotype for this species was established by Maucci and Pilato (1974) from Croatia, and the neotype for *S. grandis* **comb. nov.** – from Italy. Although those works presented new important data for both species, they do not conform to the current standards in tardigrade taxonomy. Therefore, here, we provide an integrative description for *S. spectabilis* **comb. nov.**, presenting detailed morphological data associated with DNA sequences (see Section 5.2.). Nevertheless, the validity of *S. grandis* **comb. nov.** is also supported by our phylogenetic analysis as the sequences representing the species form a distinct lineage that is sister to the cluster of sequences representing *S. spectabilis* **comb. nov.** (Fig. 1). The third species in the new genus, *Sisubiotus wuyishanensis* **comb. nov.**, was described from China. Even though the description is recent, the morphological characterisation is insufficient to differentiate the taxon from the remaining two *Sisubiotus* species. Specifically, the main character delineating *S. wuyishanensis* **comb. nov.** is supposed to be the lack of egg areolation, but the areolation is obvious in Fig. 6 in Zhang and Sun (2014). Other morphological/morphometric characters used in the differential diagnosis are minute and could be interpreted as intraspecific variability. Thus, we propose to consider *S. wuyishanensis* **comb. nov.** as *species inquirenda*.

Soon after Schuster et al. (1980) erected the genus ***Minibiotus***, its validity has been questioned by Pilato (1982) and later by Ramazzotti and Maucci (1983), who criticised it for an insufficiently clear diagnosis. Nevertheless, the genus survived the criticism and its status was strengthened by the revision of Claxton (1998) who attempted to make the genus diagnosis more precise. Currently, *Minibiotus* is no longer questioned, but the considerable morphological heterogeneity of this taxon which comprises species with and without cuticular pores, two or three macroplacoids, a single or two bends of the buccal tube, and egg processes free or enclosed in a membrane, strongly suggest that more than one genus is hiding under the name *Minibiotus* (Stec et al., 2020a). However, of the currently known 49 *Minibiotus* species, DNA sequences are available for only four named species, *Minibiotus furcatus* **stat. rev.**, *Minibiotus gumersindoi* Guil and Guidetti, 2005, *Minibiotus pentannulatus* Londoño et al., 2017, and *Minibiotus ioculator* Stec et al., 2020a, which form a paraphyletic group at the 'base' of superclade II. Although *Min. ioculator*, in contrast to the three remaining sequenced *Minibiotus* species, does not have cuticular pores, the four sequenced species do not cover the abovementioned morphological diversity and, in turn, do not yet allow for testing the phyletic nature of the genus and the relationship between morphology and phylogeny. The history of the systematic position of *Min. furcatus* **stat. rev.** is a good illustration of problems associated with the diagnosis of the genus *Minibiotus*. The species, originally described as a *Macrobiotus* by Ehrenberg in 1859 (i.e. long before *Minibiotus* was erected), was transferred to the genus *Minibiotus* by Binda and Pilato (1992) based on LCM observations of its morphology. However, recently Bertolani et al. (2014) observed that *Min. furcatus* **stat. rev.** was related more closely to *Paramacrobiotus* than to other analysed *Minibiotus* spp. in their phylogenetic analysis of Macrobiotidae; (ii) spermatozoa of *Min. furcatus* **stat. rev.** and *Paramacrobiotus* are morphologically similar; and (iii) SEM observations revealed that *Min. furcatus* **stat. rev.** has reduced peribuccal lamellae instead of papulae that are expected to characterise a *Minibiotus* species. At the same time, given that the overall morphology of *Min. furcatus* **stat. rev.** does not fit the diagnosis of *Paramacrobiotus*, Bertolani et al. (2014) moved the species tentatively back to the genus *Macrobiotus*, even though other *Macrobiotus* species clustered in remote branches of the phylogenetic tree. Finally, we retransferred *Min. furcatus* **stat. rev.** back to *Minibiotus* in the present study because: (i) it forms a clade with two *Minibiotus* species in our phylogenetic analysis (Fig. 1); (ii) as already noted by Binda and Pilato (1992), *Min. furcatus* **stat. rev.** is morphologically much more similar to *Minibiotus* species than to *Macrobiotus* species; (iii) a recent study by Stec et al. (2020a), who investigated two *Minibiotus* species with SEM, showed explicitly that their peribuccal structures, similarly to *Min. furcatus* **stat. rev.**, are not papule but shortened and thickened lamellae packed closely to each other, what questions the diagnostic value of this character in *Minibiotus*; and (iv) the similarity of male gametes of *Min. furcatus* **stat. rev.** and *Paramacrobiotus* cannot be used to exclude the species from *Minibiotus* in the absence of knowledge on spermatozoon morphology in other *Minibiotus* species. In addition to *Min. furcatus* **stat. rev.**, we also propose to transfer two *Macrobiotus* species to *Minibiotus* based on their original descriptions and LCM analysis by Fontoura et al. (2009) and a further species, *Macrobiotus spertii* Ramazzotti, 1957 that has three short macroplacoids and a closely placed microplacoid in the pharynx and egg processes equipped with a velum (please see Systematic account for details, Section 5.1.).

Tenuibiotus was established by Pilato and Lisi (2010) solely on a morphological analysis of several former *Macrobiotus* species that exhibit a characteristic claw morphology, where the primary and the secondary branch diverge at an almost right angle. Zawierucha et al. (2016), who attempted to integratively redescribe *Tenuibiotus voronkovi* (Tumanov, 2007), provided the first molecular data for the genus. These sequences were used recently by Stec et al. (2018c) and Guidetti et al. (2019) who showed a close relationship between this species and species of the genus *Paramacrobiotus*. In the present study, all *Tenuibiotus* sequences cluster in a single clade which is in a sister relationship with the *Paramacrobiotus* clade. However, it should be noted that the five species analysed herein share the most common morphotype in the genus, i.e. poreless cuticle and two macroplacoids in the pharynx, whereas there are also *Tenuibiotus* species with porous cuticle (e.g. *Tenuibiotus hyperonyx* (Maucci, 1983)) or three macroplacoids (e.g. *Tenuibiotus willardi* (Pilato, 1977)). Thus, taking into consideration that the presence of pores and the number of placoids have been shown to hold a phylogenetic signal in Macrobiotidae, it is likely that *Tenuibiotus* is polyphyletic.

The genus ***Paramacrobiotus*** was erected by Guidetti et al. (2009) based on a morphological distinction and molecular monophyly of two former species complexes within *Macrobiotus*, known as the *richtersi* and the *areolatus* group. The monophyly of the genus was later verified in other studies (Bertolani et al., 2014; Guidetti et al., 2019; Guil et al., 2019; this study). Recently, a division into two subgenera comprising species of the *richtersi* and *areolatus* groups have been proposed using a morphological criterion, i.e. the presence *vs* the absence of microplacoid, respectively (Kaczmarek et al., 2017; Marley et al. 2018). However, soon after, the subgenera were questioned by Guidetti et al. (2019) who demonstrated the polyphyly of both taxa using 18S rRNA and 28S rRNA. Most recently, Stec et al. (2020b), by a phylogenetic analysis of four genetic markers, showed that the *richtersi* group is monophyletic, but the *areolatus* group is paraphyletic, which also

questioned the subgenera. The different phyletic relationships between and within the *richtersi* and *areolatus* groups found by Guidetti et al. (2019) and by Stec et al. (2020b) suggest that a greater taxon sampling and possibly new genetic markers are needed to solve *Paramacrobiotus* phylogeny.

4.2. Morphological evolution in Macrobiotidae

The analysis of the obtained phylogenetic tree in conjunction with morphological data allowed us to discuss the evolution and taxonomic value of the key traits used in macrobiotid taxonomy: cuticle, claws, buccal apparatus, and eggs. The presence or absence of pores in the cuticle seems to bear a strong phylogenetic signal in the Macrobiotidae. Here, we analysed six genera and all, except *Minibiotus*, are monophyletic under the currently available species dataset (Fig. 1). Four of the five monophyletic genera are uniform regarding the cuticular pores and their monophyly is supported by good taxonomic sample size: *Macrobiotus* (with pores), and *Mesobiotus*, *Paramacrobiotus* and *Sisubiotus* **gen. nov.** (without pores).

Interestingly, the phylogenetic status of the two genera which both comprise a mix of species with porous and poreless cuticle, *Tenuibiotus* and *Minibiotus*, is not certain. Specifically, the fact that all five species of *Tenuibiotus* analysed in our study have no pores in the cuticle and they form a monophyletic cluster, is in line with the pattern observed in *Mesobiotus*, *Paramacrobiotus* and *Sisubiotus* **gen. nov.** Thus, we should expect that *Tenuibiotus* representatives with porous cuticle will form a separate clade. However, the lack of DNA sequences for such species currently does not allow to test this hypothesis. As noted above, *Minibiotus* is currently paraphyletic, but the poreless *Min. ioculator* clusters together with the porous *Min. furcatus* **stat. rev.** and *Min. pentannulatus* (Fig. 1), which could suggest that the presence of cuticular pores is a variable state within the genus. Moreover, when pores are present, they can vary between species by their size, shape and arrangement on the dorsal cuticle. However, given that phyletic relationships within *Minibiotus* are not resolved, the question whether the presence and shape of pores bear a phylogenetic signal cannot be answered until more species with divergent morphotypes are sequenced (Stec et al. 2020a).

Also claws seem to be phylogenetically important within this family as their morphology was used as a diagnostic criterion for the erection of *Calcarobiotus*, *Mesobiotus*, *Schusterius*, *Tenuibiotus*, and the suppressed *Xerobiotus*. However, until now, only for *Mesobiotus* and partially for *Tenuibiotus* has the monophyly been molecularly confirmed (Vecchi et al., 2016; this study). Nevertheless, the overall similarity of claws in *Calcarobiotus*, *Insuetifurca*, *Mesobiotus* and *Schusterius*, combined with the similarities of the anatomy of their buccal apparatuses (see also the paragraph below), suggest close phyletic relationships and it cannot be ruled out that some of these genera may by suppressed if molecular data show they are nested within other genera, as has been the case with *Xerobiotus* (Fig. 1). However, the example of *Xerobiotus* shows that claw morphology is not universally conservative and may be subject to intense evolutionary pressures in lineages that dwell in particular environments, such as xeric and sandy habitats, where long claws may impede locomotion. Similar adaptations have been observed also in multiple genera representing two families in Isohypsibioidea: Doryphoribiidae (*Apodibius* Dastych, 1983 and some *Doryphoribius* Pilato, 1969a) and Hexapodibiidae (*Haplohexapodibius* Pilato and Beasley, 1987, *Haplomacrobiotus* May 1948, *Hexapodibius* Pilato, 1969b, and *Parhexapodibius* Pilato, 1969a) (Bertolani and Biserov, 1996; Hohberg et al., 2011; Gąsiorek et al., 2019).

The morphology of the bucco-pharyngeal apparatus has been used in tardigrade classification since the early works concerning the phylum (Ramazzotti and Maucci, 1983). Here, we confirmed that some characteristics of the buccal apparatus, such as the number, shape and arrangement of placoids, hold a strong phylogenetic signal since all clades corresponding to genera in our analysis (Fig. 1) are also defined by unique buccal apparatus variants. Assuming that the similarities in the morphology of the buccal apparatus may be used in phylogenetic inference, we hypothesise that the following genera with three macroplacoids and a large microplacoid may be closely related: *Calcarobiotus*, *Famelobiotus*, *Insuetifurca*, *Mesobiotus* and *Schusterius*, especially considering the parallel similarities in claw morphology (see the paragraph above). On the other hand, similarly to cuticular pores, *Tenuibiotus* and *Minibiotus* are known to comprise species with different numbers of placoids in the pharynx. Furthermore, in *Minibiotus* we can find species with two bends (e.g. *Min. ioculator*, *Min. pentannulatus*) or only one bend of the buccal tube (e.g. *Min. eichhorni* Michalczyk and Kaczmarek, 2004). All this suggests the existence of multiple lineages within the two genera which may be revealed when more genetic data are available.

Egg shell variation in tardigrades is known to bear phylogenetic and taxonomic significance (Bertolani et al., 1996), both in the presence or absence of ornamentation and oviposition type (uniform within genera), as well as in details of the chorion sculpture (characteristic at species level). Our results confirmed the hypothesis formulated in Guidetti et al. (2013) that chorion ornamentation evolves faster than animal morphology. The most widespread model of egg ornamentation in Macrobiotidae is chorion equipped with cone-shaped processes which is present in all macrobiotid genera in which the eggs are known (eggs are unknown for six of the fourteen described genera: *Biserovus*, *Famelobiotus*, *Insuetifurca*, *Minilentus*, *Pseudohexapodibius*, and *Schusterius*). Furthermore, the cone-shape processes are present in all Richtersiidae and Murrayidae, excluding only *Adorybiotus*, which are sister to Macrobiotidae and are used in our analysis as the outgroup. Thus, it is likely that the ancestor of Macrobiotidae laid eggs with cone-shaped processes. If this was indeed the case, then cone-shaped processes with branched or filamentous apices (in some species of *Calcarobiotus*, *Minibiotus*, *Mesobiotus* and *Tenuibiotus*) and mushroom-shaped processes (in many *Macrobiotus s.s.* species) are derived morphotypes. Whereas the first type of egg processes probably evolved independently several times, mushroom-shaped processes are present only within *Macrobiotus s.s.* and are exhibited by the majority of its species (Fig. 1), thus they are likely to be a symplesiomorphic state for the genus. However, the presence of species groups within *Macrobiotus s.s.* which are characterised by areolated eggs with conical processes (Fig. 8) that resemble eggs of the distantly related *Paramacrobiotus*, is an explicit example of the evolutionary plasticity of the egg shell ornamentation in the genus *Macrobiotus s.s.* If mushroom-shaped processes are indeed the ancestral state for *Macrobiotus s.s.*, then the reversal to *Paramacrobiotus*-like eggs in the *Mac. pallari* and *Mac. nelsonae* complexes is truly remarkable, especially given the convergent evolution of the labyrinthine layer within the process walls (walls without the layer in mushroom-shaped processes; Fig. 8). Whereas the labyrinthine layer in species of the *Mac. nelsonae* complex forms *Paramacrobiotus*-like reticulation, the morphology of the layer in the *Mac. pallarii* complex exhibits interesting variation. Specifically, from well-developed reticulation in *Mac. pallarii*, *Mac. ragonesei* Binda et al., 2001, and the Montenegrin *Mac.* cf. *pallarii* population (ME.007, Fig. 8) through an intermediate state with reticulation visible only in the bottom part of egg processes in the Polish and the Finnish *Mac.* cf. *pallarii* populations (FI.066 and PL.015), to scattered bubbles in the American *Mac.* cf. *pallarii* population (US.057) and *Mac. caymanensis* Meyer, 2011 (see also figures 17–18 in Meyer 2011). Another important aspect of egg ornamentation, in the context of macrobiotid evolution, is the morphology of chorion surface (between egg processes) which exhibits a considerable variation across the family and several general states can be distinguished: solid ("smooth"), porous, and reticulated chorion surface, but also surface covered by areolation or semi-areolation formed by finger-like expansions radiating from process bases. Although areolation is found in several distinct macrobiotid lineages, it is the only of the five chorion surface states listed above that characterises some genera (i.e. *Paramacrobiotus* and *Sisubiotus* **gen. nov.**), whereas in other genera more than one state is present. The most drastic example is the genus *Mesobiotus*, in which all mentioned morphotypes are present (Kaczmarek et al. 2020). Similarly to egg

processes, this extreme diversity in chorion surface also exemplifies the dynamics of the morphological evolution in Macrobiotidae. Similar incongruencies between taxonomically important traits (i.e. useful in species delineation and identification) and phylogeny were recently found in two distantly related genera, *Milnesium* (order Apochela) and *Bryodelphax* (class Heterotardigrada), by Morek and Michalczyk (2020) and Gąsiorek et al. (2020), respectively. This may suggest that such a mosaic pattern of morphological evolution is common in tardigrades. If this is indeed true, then more genera that were established with the sole use morphology, such as *Xerobiotus*, may turn out to be invalid.

Although this study presents the largest and most detailed phylogenetic analysis of the family Macrobiotidae to date, there is still a plethora of questions to be answered regarding the phyletic affinities, morphological evolution and systematics of this super-diverse tardigrade group. We would like to think that we start to see the picture painted by nature, but considering that over 80% of the known macrobiotid species have not yet been sequenced, including many that represent unique morphotypes, and given that many more species are awaiting to be discovered, the systematics of the family could be far from stable and may undergo significant changes in the future.

5. Systematic account

5.1. *Macrobiotus* taxonomy

Phylum: Tardigrada Doyère, 1840
Class: Eutardigrada Richters, 1926
Order: Parachela Schuster et al., 1980 (restored by Morek et al., 2020)
Superfamily: Macrobiotoidea Thulin, 1928
Family: Macrobiotidae Thulin, 1928
Genus: *Macrobiotus* C.A.S. Schultze, 1834 (emended diagnosis)

Diagnosis: Macrobiotidae with: (*i*) porous cuticle, (*ii*) mouth opening surrounded by ten peribuccal lamellae, (*iii*) a rigid buccal tube strengthened with the ventral lamina lacking a ventral hook, (*iv*) two elongated macroplacoids and a microplacoid positioned close to them, (*v*) Y-shaped claws of the *hufelandi* type with lunulae on each leg or claws are reduced and devoid of lunulae (only in the *Mac. pseudohufelandi* complex), (*vi*) eggs with an ornamented shell laid freely to the environment.

Remarks:. *All characters presented in the original description of the following four species of Macrobiotus meet the taxonomic criteria of the genus Minibiotus (see Discussion for more details) and are thus transferred to the latter genus*: *Minibiotus furcatus* (Ehrenberg, 1859) **stat. rev.**, *Minibiotus pseudofurcatus* (Pilato, 1972), **comb. nov.** *and Minibiotus lazzaroi* (Maucci, 1986) **comb. nov.**, *Minibiotus spertii* (Ramazzotti, 1957) **comb. nov.**

Based on the genetic and morphological evidence presented in this study, the three currently recognised species of *Xerobiotus* are transferred to *Macrobiotus s.s.* (see Discussion for more details): *Macrobiotus pseudohufelandi* (Iharos, 1966a, 1966b) **stat. rev.**, *Macrobiotus xerophilus* (Dastych, 1978) **stat. rev.** and *Macrobiotus euxinus* (Pilato et al., 2011) **comb. nov.**

Because of highly insufficient descriptions preventing a confident identification (often the lack of information on the key traits that are currently used to differentiate the species, such as leg granulation and/or lunule morphology, and/or oral cavity armature, and/or morphometric characters, and/or vague description of the egg ornamentation

Table 3
Measurements [in µm] of selected morphological structures of individuals from the Finnish population of *Sisubiotus spectabilis* (Thulin, 1928) **comb. nov.** mounted in Hoyer's medium (N–number of specimens/structures measured, RANGE refers to the smallest and the largest structure among all measured specimens; SD–standard deviation).

CHARACTER	N	RANGE						MEAN		SD	
		µm			*pt*			µm	*pt*	µm	*pt*
Body length	28	443	–	989	*907*	–	*1276*	720	*1109*	122	*91*
Buccal tube											
Buccal tube length	28	40.8	–	77.5		–		64.7	–	8.1	–
Stylet support insertion point	28	32.6	–	62.5	*78.5*	–	*81.9*	52.0	*80.3*	6.7	*1.0*
Buccal tube external width	28	7.0	–	15.9	*17.0*	–	*22.1*	12.4	*19.0*	2.2	*1.5*
Buccal tube internal width	28	5.0	–	12.7	*12.3*	–	*17.6*	9.6	*14.7*	1.9	*1.5*
Ventral lamina length	27	24.5	–	47.1	*54.8*	–	*64.8*	39.4	*60.9*	5.2	*2.3*
Placoid lengths											
Macroplacoid 1	28	12.9	–	25.0	*24.7*	–	*35.3*	20.0	*30.8*	3.3	*2.4*
Macroplacoid 2	28	8.6	–	19.7	*17.6*	–	*26.0*	14.3	*21.9*	2.8	*2.2*
Microplacoid	28	4.2	–	8.9	*8.6*	–	*12.6*	6.6	*10.2*	1.2	*1.1*
Macroplacoid row	28	24.1	–	46.6	*49.6*	–	*63.0*	37.5	*57.8*	6.2	*3.5*
Placoid row	28	29.6	–	55.7	*59.1*	–	*77.6*	45.6	*70.3*	7.3	*3.9*
Claw 1 heights											
External primary branch	26	11.1	–	20.9	*22.0*	–	*29.2*	17.0	*26.0*	2.5	*1.9*
External secondary branch	24	8.6	–	16.5	*17.3*	–	*22.7*	13.0	*20.0*	2.1	*1.8*
Internal primary branch	26	10.6	–	19.8	*19.7*	–	*28.4*	16.0	*24.5*	2.5	*2.0*
Internal secondary branch	25	8.2	–	15.6	*15.4*	–	*22.0*	12.4	*19.1*	2.0	*1.8*
Claw 2 heights											
External primary branch	25	12.4	–	22.8	*24.3*	–	*36.8*	18.2	*28.4*	2.9	*2.7*
External secondary branch	24	9.2	–	18.0	*18.3*	–	*29.2*	14.1	*21.9*	2.4	*2.4*
Internal primary branch	24	10.2	–	20.0	*22.0*	–	*30.3*	16.2	*25.3*	2.5	*1.7*
Internal secondary branch	23	7.6	–	16.5	*17.1*	–	*26.7*	12.9	*20.1*	2.3	*2.1*
Claw 3 heights											
External primary branch	25	12.2	–	22.9	*21.7*	–	*31.7*	18.2	*28.0*	2.7	*2.2*
External secondary branch	22	8.9	–	17.7	*17.4*	–	*24.5*	14.0	*21.6*	2.3	*1.8*
Internal primary branch	24	11.4	–	20.9	*21.5*	–	*28.9*	16.5	*25.4*	2.5	*2.0*
Internal secondary branch	21	8.7	–	16.9	*17.3*	–	*23.4*	12.9	*19.9*	2.2	*1.8*
Claw 4 heights											
Anterior primary branch	25	13.4	–	26.6	*27.3*	–	*36.8*	21.1	*32.5*	3.5	*2.7*
Anterior secondary branch	24	9.1	–	19.0	*19.6*	–	*26.3*	15.2	*23.3*	2.4	*1.8*
Posterior primary branch	25	14.6	–	27.2	*29.8*	–	*38.8*	22.5	*34.7*	3.5	*2.6*
Posterior secondary branch	23	11.2	–	19.8	*21.7*	–	*27.9*	16.4	*24.9*	2.3	*1.9*

morphology), the following species are considered as *nomina inquirenda*: *Macrobiotus annae* Richters, 1908 **nom. inq.**, *Macrobiotus ascensionis* (Richters, 1908) **nom. inq.**, *Macrobiotus brevipes* Mihelčič, 1971/72 **nom. inq.**, *Macrobiotus carsicus* Maucci, 1954 **nom. inq.**, *Macrobiotus evelinae* de Barros, 1938 **nom. inq.**, *Macrobiotus gemmatus* Bartoš, 1963 **nom. inq.**, *Macrobiotus hibiscus* de Barros, 1942 **nom. inq.**, *Macrobiotus insignis* Bartoš, 1963 **nom. inq.**, *Macrobiotus kolleri* Mihelčič, 1951 **nom. inq.**, *Macrobiotus komareki* Bartoš, 1939 **nom. inq.**, *Macrobiotus longipes* Mihelčič, 1971/72 **nom. inq.**, *Macrobiotus ovidii* Bartoš, 1937 **nom. inq.**, *Macrobiotus ovovillosus* Baumann, 1960 **nom. inq.**, *Macrobiotus papillosus* Iharos, 1963 **nom. inq.**, *Macrobiotus porteri* Rahm, 1931 **nom. inq.**, *Macrobiotus potockii* Węglarska, 1968 **nom. inq.**, *Macrobiotus rollei* Heinis, 1920 **nom. inq.**, *Macrobiotus striatus* Mihelčič, 1949 **nom. inq.**, *Macrobiotus terricola* Mihelčič, 1951 **nom. inq.**, *Macrobiotus tetraplacoides* Fontoura, 1981 **nom. inq.**, *Macrobiotus topali* Iharos, 1969 **nom. inq.**, *Macrobiotus virgatus* Murray, 1910 **nom. inq.**

Due to highly insufficient descriptions that lack many key traits used currently in species differentiation (listed above) and because of the doubtful descriptions of eggs that are deposited in exuviae what is atypical for the genus and the family, the following species are not identifiable and are considered as *nomina dubia*: *Macrobiotus artipharyngis* Iharos, 1940 **nom. dub.**, *Macrobiotus norvegicus* Mihelčič, 1971/72 **nom. dub.**, *Macrobiotus rubens* Murray, 1907 **nom. dub.**

Because of highly insufficient descriptions that lack many key traits used currently in species differentiation (listed above) and because of the lack of eggs description, which are most often crucial for species identification in Macrobiotidae, the following species are not identifiable and are considered as *nomina dubia*: *Macrobiotus shennongensis* Yang, 1999 **nom. dub.**, *Macrobiotus yunshanensis* Yang, 2002 **nom. dub.**

All characters of animals and eggs presented in the original description of *Macrobiotus caelicola* Kathman, 1990 meet the morphological criteria of *Diaforobiotus* Guidetti et al., 2016 (Eutardigrada: Richtersiidae), thus it is transferred to the genus with the following combination: *Diaforobiotus caelicola* (Kathman, 1990) **comb. nov.**

Table 4
Measurements [in μm] of selected morphological structures of the eggs from the Finnish population of *Sisubiotus spectabilis* (Thulin, 1928) **comb. nov.** mounted in Hoyer's medium (N–number of eggs/structures measured, RANGE refers to the smallest and the largest structure among all measured specimens; SD–standard deviation).

CHARACTER	N	RANGE			MEAN	SD
Egg bare diameter	7	93.5	–	121.5	107.8	11.1
Egg full diameter	7	146.2	–	213.5	175.4	22.1
Process height	72	23.8	–	47.4	32.6	5.1
Process base width	72	14.7	–	29.8	23.0	3.4
Process base/height ratio	72	40%	–	111%	73%	17%
Inter-process distance	72	3.9	–	13.7	8.2	2.0
Number of processes on the egg circumference	7	10	–	12	11.3	0.8

5.2. *Integrative description of new populations of Sisubiotus spectabilis (Thulin, 1928) comb. nov.*

(Tables 3–4, Figs. 2–7)

5.2.1. *Material examined:*

45 animals, and 38 eggs. Specimens mounted on microscope slides in Hoyer's medium (36a + 26e), fixed on SEM stubs (5a + 10e), processed for DNA sequencing (2a + 2e from the Finnish population and 2a from the Norwegian population).

5.2.2. *Localities of two new populations examined in this study:*

62°13′45.8″N, 25°44′39.5″E, 95 m asl: Finland, Jyväskylä,

Table 5
Species complexes (clades clustering morphologically similar species) and the *Macrobiotus hufelandi* morphogroup (a polyphyletic group of morphologically similar species) within the monophyletic *Macrobiotus s.s.*. Regardless of the phylogenetic status of the groups listed in the table, they are all useful taxonomically. Monophyly states: + = monophyly confirmed by a phylogenetic analysis based on the currently available data (i.e. new sequences may negate the monophyly), – = lack of monophyly confirmed by a phylogenetic analysis, (+) = species in a polytomy but within a single clade, thus monophyly possible when sequences for additional species strengthen the statistical support. Underlined are species for which genetic data are available and their affiliation (or lack thereof) to particular species complexes was phylogenetically confirmed in the present study. *Remarks: Macrobiotus ariekammensis* and *Macrobiotus polyopus* groups (mentioned in the Discussion Section 4.1.) are not included in the table as genetic data for them are unavailable, thus their phylogenetic character and affinities are not known.

Species complex/ morphogroup	Description	Species (alphabetically)	Monophyly
Macrobiotus nelsonae complex	Species with white body, *hufelandi* type claws and with eggs with conical processes (with the labyrinthine layer visible as reticulation under LCM) separated by two rows of areolae.	*deceptor**, *nelsonae*,cf. *nelsonae* (in Bertolani et al. 2014)	(+)
Macrobiotus pallarii complex	Species with white body, *hufelandi* type claws and with eggs with conical processes (with the labyrinthine layer visible as reticulation or bubbles under LCM) separated by one row of areolae.	*caymanensis*, *pallarii*, cf. *pallarii* (ME.007**, in this study), cf. *pallarii* (US.057**, in this study), cf. *pallarii* (PL.015/FI.066**, in this study), *ragonesei*	+
Macrobiotus persimilis complex	Species with white body, *hufelandi* type claws and with single-walled egg processes (without the labyrinthine layer = not reticulated) in the shape of truncated cones terminated with a well-developed disc and with solid chorion surface (wrinkled but never porous or reticulated).	*anemone*, *caelestis*, *dulciporus*, *engbergi*, *halophilus*, *hyperboreus*, *marlenae*, *patagonicus*, *persimilis*, *polonicus*, *trunovae*,	+
Macrobiotus pseudohufelandi complex (former *Xerobiotus*)	Species with white body, strongly reduced claws on all legs and with lunulae present only on the hind legs.	*pseudohufelandi* **stat. rev.**, *xerophilus* **stat. rev.**, *euxinus* **comb. nov.**, aff. *pseudohufelandi* PL.360 (in this study), aff. *pseudohufelandi* ZA.373 (in this study)	+
Macrobiotus hufelandi morphogroup	All species that do not fall under any of the four species complexes listed above, i.e. species with white or yellowish body, *hufelandi* type claws and with single-walled egg processes (without the labyrinthine layer = not reticulated): (i) in the shape of truncated cones terminated with a disc, or (ii) in the shape of cones, sometimes elongated into flexible filaments or spatula, or (iii) in the shape of pegs; with egg surface mostly reticulated or porous, but when solid, the processes are of a modified shape (e.g. terminal discs with flexible filaments or elongated into filaments or a spatula).	*almadai*, *biserovi*, *canaricus*, *dariae*, *denticulus*, *diversus*, *glebkai*, *horningi*, *hufelandi*, *hannae*, *humilis*, *iharosi*, *joannae*, *julianae*, *kamilae*, *kazmierskii*, *kristenseni*, *lissostomus*, *macrocalix*, *maculatus*, *madegassus*, *martini*, *modestus*, *naskreckii*, *nebrodensis*, *noemiae*, *noongaris*, *papei*, *paulinae*, *personatus*, *polypiformis*, *punctillus*, *ramoli*, *rawsoni*, *recens*, *sandrae*, *sapiens*, *scoticus*, *serratus*, *semmelweisi*, *seychellensis*, *shonaicus*, *sottilei*, *terminalis*, *wandae*, *vladimiri*	–

* – Provisional assignment as cuticular pores have to be confirmed with SEM analysis.
** – Species under description in a separate paper.

Survontie; moss on a rock between roadside and forest; coll. 07.03.2019 by Matteo Vecchi.

62°10′33.24″N, 9°27′5.22″E, 943 m asl: Norway, Vicinity of lake Avsjøen and E8 route; mixed moss and lichen sample on a rock in a shrubland; coll. 23.07.2017 by Daniel Stec and Witold Morek (only two animals have been found in this sample and they were used for molecular analysis).

5.2.3. Slide and SEM stubs depositories:

36 animals (slides: FI.067.*, where the asterisk can be substituted by any of the following numbers 05–08, 13–16, 18) and 26 eggs (slides: FI.067.*: 09–12, 17) as well as SEM stub: 18.11 are deposited at the Institute of Zoology and Biomedical Research, Jagiellonian University, Gronostajowa 9, 30–387, Kraków, Poland.

*5.2.4. **Animals** (measurements and statistics in Table 4):*

In live animals, body almost transparent in smaller specimens and white in larger animals; after fixation in Hoyer's medium body transparent (Fig. 2A). Eyes present in live animals and after fixation in Hoyer's medium. Cuticle poreless but dorsal cuticle covered with fine granulation visible only in SEM (Fig. 2B). Patches of fine granulation on the external surface of legs I–III as well as dorsal and dorso lateral of legs IV clearly visible in LCM (Fig. 2C, F) and SEM (Fig. 2E, H). The granulation on legs IV is composed of larger granules (microgranule aggregations) near the claws, but the granulation decreases proximally in size and gradually becomes the fine granulation that covers the entire dorsal cuticle (Fig. 2H). Leg granulation is composed of microgranule aggregations (Fig. 2F, H). A pulvinus is present on the internal surface of legs I–III (Fig. 2D, G).

Claws slender, of the *hufelandi* type. Primary branches with distinct accessory points, a long common tract, and with an evident stalk connecting the claw to the lunula (Fig. 3A–D). The end of the common tract is constricted in all claws but this is clearly visible in SEM and only sometimes in LCM (Fig. 3A, C–D). All lunulae smooth (Fig. 3A–D). Paired muscle attachments on legs I–III often very visible both in LCM and SEM (Fig. 3A, C), whereas the horseshoe-shaped structure under claws IV poorly visible only in LCM (Fig. 3D) but not in SEM.

Mouth antero-ventral. Bucco-pharyngeal apparatus of the *Macrobiotus* type (Fig. 4A), with the ventral lamina and ten small peribuccal lamellae. The oral cavity armature well developed and composed of three bands of teeth, always clearly visible under LCM (Fig. 4B–C). The first band of teeth is composed of numerous small teeth visible in LCM as granules (Fig. 4B–C) and as cones in SEM (Fig. 5A–B), arranged in several rows, situated anteriorly in the oral cavity, just behind the bases of the peribuccal lamellae. The second band of teeth is situated between the ring fold and the third band of teeth and comprises 3–4 rows of teeth visible in LCM as granules (Fig. 4B–C) and as cones in SEM (Fig. 5A–B), but larger than those in the first band. The most anterior row of teeth within the second band comprises larger and longitudinally elongated teeth than the subsequent posterior rows (Fig. 4B–C and 5A–B). 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. 4B–C and 5A–B). The third band of teeth is divided into the dorsal and the ventral portion. Under both LCM and SEM, the dorsal teeth are seen as three distinct transverse ridges whereas the ventral teeth appear as two separate lateral transverse ridges between which one big tooth is visible (Fig. 4B–C). In SEM, ventral teeth are blunt whereas the dorsal teeth have indented and sharp margins (Fig. 5A–B). Pharyngeal bulb spherical, with triangular apophyses, two rod-shaped macroplacoids and a large microplacoid positioned close to them (i.e. the distance between the second macroplacoid is shorter than the microplacoid length; Fig. 4D–E). The macroplacoid length sequence is $2 < 1$. The first macroplacoid is anteriorly narrowed and constricted in the middle whereas the second has a sub-terminal constriction (Fig. 4E–F).

*5.2.5. **Eggs** (measurements and statistics in Table 5):*

Laid freely, white, spherical with large conical processes, areolated (Fig. 6A–B and 7A). The labyrinthine layer between the process walls absent, i.e. matrix between the external and internal wall homogenous (Fig. 6A–G). The upper part of process surface is covered, to a varying extent, by microgranulation visible only under SEM (Fig. 7D–F). Each process is surrounded by ten to fourteen deep, areolae. Usually two rows of areolae are present between the neighbouring processes, but sometimes only a single row connects the processes (Fig. 6C–D, 7A–D). Areolae rims thin and high, covered with micropores (visible only in SEM; Fig. 7D). In SEM, areola surface is reticulated (Fig. 7A–D), but under LCM only the knots of the mesh are visible as dots (Fig. 6C–D).

5.2.6. Reproduction

The examination of animals freshly mounted in Hoyer's medium revealed the lack of testis or spermathecae filled with spermatozoa in all of the analysed specimens.

6. Conclusions

Our study, by increasing species sample size in reconstructing phylogeny in comparison with earlier works, sheds new light on the evolution of Macrobiotidae. One of the discovered lineages is elevated to the genus level, *Sisubiotus* **gen. nov.** We also confirmed the monophyly for five out of the six studied genera which are additionally discussed and revised in our work. Our results further indicate that special effort should be made towards increasing sample size for the genus *Minibiotus* but also for the remaining, rarely found macrobiotid genera. Increasing taxonomic sample size will most probably lead to the discovery of new distinct evolutionary lineages within the family Macrobiotidae.

CRediT authorship contribution statement

Daniel Stec: Conceptualisation, Methodology, Software, Data curation, Writing - original draft, Visualisation, Investigation, Writing - review & editing, Funding acquisition, Formal analysis. **Matteo Vecchi:** Methodology, Software, Data curation, Writing - original draft, Visualisation, Formal analysis. **Sara Calhim:** Writing - original draft, Supervision. **Łukasz Michalczyk:** Conceptualisation, Writing - original draft, Visualisation, Writing - review & editing, Funding acquisition, Supervision.

Acknowledgments

We are especially grateful to our colleagues Aneta and Marcus Rumler, Aleksandra Rysiewska, Bartłomiej Surmacz, Krzysztof Miler, Nate Gross, Mackenzie McClay, Paul Bartels, Peter Degma, Piotr Gąsiorek, Witold Morek for collecting and sending samples which enable us to conduct this study. We are also grateful to Dr. Simon Carol for her invaluable help in sampling in the Republic of South Africa. Moreover, we thank to Dr. Roberto Guidetti and the Civic Museum of Natural History of Verona for making the Maucci collection available. Samples from the Republic of South Africa were collected under permits No: CN35-285316 issued by CapeNature to Witold Morek. The study was supported by the *Preludium* programme of the Polish National Science Centre (grant no. 2018/31/N/NZ8/03096 to DS supervised by ŁM). During this study, DS was a beneficiary of a National Science Centre scholarship to support doctoral research (no. 2019/32/T/NZ8/00348). Some of the analyses were carried out with the equipment purchased from the *Sonata Bis* programme of the Polish National Science Centre (grant no. 2016/22/E/NZ8/00417 to ŁM). Open-access publication of this article was funded by the BioS Priority Research Area under the program "Excellence Initiative – Research University" at the Jagiellonian University in Kraków, Poland.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2020.106987.

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