Integrative Redescription of the *Minibiotus intermedius* (Plate, 1888)—The Type Species of the Genus *Minibiotus* R.O. Schuster, 1980

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Abstract: In the present study, we used the integrative taxonomy approach to redescribe *Minibiotus intermedius* based on the newly found topotypic population in Marburg (Germany). As the original type material is not available, we designate a neotype to stabilize the taxonomy of the genus *Minibiotus*. Obtained mitochondrial COI barcode sequence and nuclear markers, i.e., 18S rRNA and 28S rRNA of *M. intermedius* from the neotype locality, were unique and distinct from those deposited in GenBank. In the first redescription of *M. intermedius*, only four specimens and no eggs from the neotype locality were analyzed. Moreover, genetic analyses were not conducted and barcodes were not available. Therefore, the present study, by establishing the neotype and providing integrative data on the neotype population, helps to better define the *Minibiotus* taxonomy and prevents further misunderstandings in the future.

Keywords: Europe; Eutardigrada; Germany; Macrobiotidae; Tardigrada; taxonomy

1. Introduction

Tardigrades inhabit aquatic (freshwater and marine) and terrestrial habitats, from the highest mountain peaks to deepest oceans, from the polar regions to the tropics. They are found in lichens, mosses, leaf litter, soil, sediments, and on aquatic plants [1]. Up to now, about 1400 species of hetero and eutardigrades have been described throughout the world [2–5].

The genus *Minibiotus* R.O. Schuster [6] was established more than forty years ago by Schuster et al. [6]. However, at first, the new genus was not easily accepted by other researchers who questioned its validity due to an insufficiently clear diagnosis [7,8]. Later, Claxton [9] published the most comprehensive revision of this genus based on animal and egg morphology, strengthening the generic diagnosis and, at the same time, its validity. In that time, 22 species were included into the genus *Minibiotus*; however, until now, many new species have been described. According to the current tardigrade checklist, 48 species are known in this genus [5]. The genus is mainly characterized by their antero-ventral mouth surrounded by 10 papulae, a narrow buccal tube with either a single or a double
curvature, short ventral support, two or three short macroplacoids, and short macroplacoid row length [10]. However, recently, Stec et al. [10,11] stated that genetic data for the genus are very scarce (i.e., only 39 *Minibiotus* DNA sequences deposited in GenBank and many of them are not identified to species level), and also that detailed high-resolution data on the genus morphology are extremely limited. Up to now, genetic sequences are available for four nominal *Minibiotus* taxa: *M. furcatus* (Ehrenberg [12]), *M. gumersidoi* (Guil and Guidetti [13]), *M. ioculator* (Stec, Kristensen, and Michalczyk [10]), and *M. pentanulatus* (Londoño, Daza, Lisi, and Quiroga [10,14–16]). The available DNA sequences and extreme morphological diversity suggest that *Minibiotus* is probably polyphyletic and some species are closely related to *Paramacrobiotus* Guidetti et al. [16–18]. The mentioned phenotypic diversity of the genus accounts for the presence or absence of pores in the animal cuticle, egg processes enclosed within or without membrane, and bucco-pharyngal apparatuses with two or three macroplacoids [18,19]. The hypothesis on *Minibiotus* polyphyly based on the morphological diversity present in the genus is further supported by the recent study by Stec and Morek [20], who found and clarified a similar situation within the genus *Tenuibiotus* (Pilato and Lisi [21]). This study demonstrated that such morphological characteristics might generally be considered conservative and stable at the genus level. Importantly, beside the parapthyly problem in the genus *Minibiotus*, the more pressing issue concerns the insufficient description and characterization of the type species for the genus—*M. intermedius* (Plate [22]). As shown by several recent studies, integrative redescriptions of such important taxa are crucial in opening the widow for more precise quantification and description of tardigrade species diversity (e.g., [23–33]). Therefore, the correct and detailed redescription of type taxa is drastically needed.

In this paper, we provide an integrative redescription of *M. intermedius* from its original type locality in Marburg (Germany). The redescription is based on detailed phenotypic data of animals and eggs collected with the use of light and scanning electron microscopy. This morphological information is tightly associated with genetic data in the form of DNA sequences of three molecular markers (18S rRNA, 28S rRNA and COI).

2. Materials and Methods

2.1. Sampling

A single sample of mosses and lichens from a tree was collected in a mixed forest in Marburg (Germany) in September 2019 (sample code (SC) GR2, for more details see below). The sample was then packed in a paper envelope; dried at a temperature of ca. 20 °C; and delivered to the Department of Animal Taxonomy and Ecology at the Faculty of Biology, Adam Mickiewicz University in Poznań, Poland. The tardigrade collection, extraction, and mounting techniques followed the protocol of Dastych [34].

2.2. Microscopy and Imaging

A total of 38 specimens and eight eggs were mounted on microscope slides in Hoyer’s medium, and then examined under an Olympus BX41 Phase Contrast light Microscope (PCM) associated with an Olympus SC50 digital camera (Olympus Corporation, Shinjuku-ku, Japan).

Three eggs were prepared for scanning electron microscope (SEM) analysis according to the protocol in Roszkowska et al. [35] and examined under a high vacuum in Hitachi S3000N SEM.

All figures were assembled in Corel Photo-Paint 2017. For deep structures that could not be fully focused in a single photograph, a series of 2–50 images was taken every ca. 0.5 μm depth and then manually assembled into a single deep-focus image in Corel Photo-Paint 2017.
2.3. Morphometrics and Morphological Nomenclature

All measurements are given in micrometres [µm]. The sample size was adjusted following recommendations by Stec et al. [36]. 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 type of bucco-pharyngeal apparatus and claws was classified according to Pilato and Binda [37]. The terminology used to describe oral cavity armature and egg shell morphology follows Michalczyk and Kaczmarek [38] and Kaczmarek and Michalczyk [39]. Macroplacoid length sequence is given according to Kaczmarek et al. [40]. The buccal tube length and the level of the stylet support insertion point were measured according to Pilato [41]. The pt index is the ratio of the length of a given structure to the length of the buccal tube expressed as a percentage [41]. Cuticular bars and muscle attachments under claws were classified according to Kiosya et al. [42]. All other measurements and nomenclature follow Kaczmarek and Michalczyk [39]. Morphometric data were handled using the “Parachela” ver. 1.8 template available from the Tardigrada Register [43]. Raw morphometric data for each analyzed species are provided as Supplementary Materials (SM. 1). Tardigrade taxonomy follows Bertolani et al. [16] and Stec et al. [18].

2.4. Genotyping

Three specimens of M. intermedius (isolates numbers: Min3GR, Min4GR, and Min6GR) were preliminarily identified in vivo using light microscopy (LM) prior to DNA extraction for genotyping analysis. Genomic DNA was extracted from individual animals using a Chelex® 100 resin (Bio-Rad) method [44] with modifications described in detail in Kaczmarek et al. [45]. The tardigrade exoskeleton was extracted from a pellet containing Chelex beads on the bottom of each tube. Obtained exoskeletons were mounted on a microscope slide in Hoyer’s medium for further morphological analysis and deposited in the collection of the Department of Animal Taxonomy and Ecology, Faculty of Biology, Adam Mickiewicz University in Poznań (Poland).

We sequenced three DNA fragments with different mutation rates. A fragment of the cytochrome oxidase subunit I (COI, mtDNA) was amplified using universal primers: HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') and LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') [46]. In turn, the cytoplasmic ribosome small (18S rRNA, nDNA) and large (28S rRNA, nDNA) subunit components were amplified using the following primers: SSU01_F (5'-AACCTGGTTGATCCTGCCAGT-3') and SSU82_R (5'-TGATCTCTTGAGGCTACCCCTATAC-3') [47] for the 18S rRNA sequences and 28SF0001 (5'-ACCvCynAATTTAAGCATAT-3') and 28SR0990 (5'-CCTTGGTCCGTGTTTCAAGAC-3') [48] for the 28S rRNA sequences. For every PCR reaction, the solution contained 7.5 µL of ddH2O, 0.5 µL of 5 µM forward primer, 0.5 µL of 5 µM reverse primer, 10 µL of JumpStart™ Taq ReadyMix™ DNA polymerase (Sigma-Aldrich™), and 1.5 µL of genomic DNA extract. The PCR protocols for amplification of the mitochondrial COI gene fragment and nuclear 18S rRNA sequences, agarose gel electrophoresis, and sequencing were performed according to Mioduchowska et al. [49]. In turn, the PCR cycling profile to amplify the 28S rRNA gene fragment was as follows: initial denaturation at 95 °C for 5 min followed by 40 cycles of 95 °C for 30 s, 50 °C for 1 min, and 72 °C for 90 s, ending with 72 °C for 5 min.

2.5. Comparative Molecular Analysis

Obtained mitochondrial and nuclear sequences were checked for quality and were manually aligned in BioEdit v. 7.2.5 [50]. Basic local alignment search tool (BLAST) [51] searches were performed to verify the identity and homology of the obtained gene fragments with sequences deposited in the NCBI database. The COI sequence was translated into amino acid sequences with the invertebrate mitochondrial codon table and the 2th reading frame using the EMBOSS-TRANSEQ application [52,53].
For molecular comparisons, all sequences of species belonging to the genus *Minibiotus* were downloaded from the GenBank database and aligned using the ClustalW multiple alignment tool [54], implemented in BioEdit v. 7.2.5. Only the available GenBank sequences that represented a homologous fragment with nrDNA and mtDNA sequences obtained in our study were applied. Alignment sequences were trimmed to 472, 1138, and 678 bp for COI (13 sequences), 18S rRNA (6 sequences), and 28S rRNA (10 sequences) molecular markers, respectively. The uncorrected \( p \)-distances were calculated using pairwise deletion for the gap/missing data treatment option and the software MEGA X [55]. Detailed \( p \)-distance tables are presented as Supplementary Materials (SM.02).

Phylogenetic trees were computed using the software MEGA X by applying maximum likelihood (ML) analysis under the general settings of selected models with 1000 bootstraps. The best-fit substitution models were determined using jModelTest v. 2.1.4 [56] with the assumptions of both the Bayesian inference criterion (BIC) and the Akaike information criterion (AIC) [57]: the Hasegawa–Kishino–Yano with gamma (HKY + G) distribution (G parameter = 0.1899) [58] for COI sequences, the Kimura 2-parameter (K2) model [59] for 18S rRNA sequences, and the Kimura 2-parameter (K2 + G) model with gamma distribution (G parameter = 0.1709) for 28S rRNA sequences. The molecular markers of *Macrobiotus porifini* (GenBank accession numbers: COI–MT246659, 18S rRNA–MT241900, 28S rRNA–MT241897 [60]) were used as outgroups. Initial evolutionary trees for the heuristic search were generated by applying BioNJ and neighbor join algorithms to a matrix of pairwise distances (which was estimated using the maximum composite likelihood (MCL) approach). Finally, the topology of phylogenetic trees was selected with superior log likelihood value and visualized by FigTree v.1.4.3 and Inkscape v.0.92.

All obtained sequences were deposited in GenBank under the following accession numbers: COI–ON005160, 18S rRNA–ON005188-8,9 and 28S rRNA–ON005193-95 (see also SM.02).

3. Results
3.1. Taxonomic Account

- Phylum: Tardigrada Doyère [61]
- Class: Eutardigrada Richters [62]
- Order: Parachela Schuster, Nelson, Grigarick, and Christenberry [6]
- Superfamily: Macrobiotoidea Thulin [63] (in [64])
- Family: Macrobiotidae Thulin [63]
- Genus: *Minibiotus* R.O. Schuster [6] (in [6])

*Minibiotus intermedius* (Plate [22])

(Tables 1 and 2, Figures 1–5).

*Neotype locality:* Germany, 50°47′49″ N, 08°46′45″ E, 247 m asl, State of Hessen, Marburg, mosses and lichens from tree, mixed forest, 2 September 2019, coll. Johenn Sholl.

*Material examined:* 38 specimens and 8 eggs, i.e., neotype + 45 neoparatypes (37 specimens and 8 eggs) mounted on microscope slides in Hoyer’s medium, 3 eggs prepared for SEM, and 10 specimens prepared for molecular analyses. However, DNA sequences were obtained only from three specimens (isolates numbers Min3GR, Min4GR, and Min6GR).

*Type depositories:* Neotype (slide GR2/12), 35 neoparatypes (slides GR 2/2, and GR2/4–2/14) are deposited at the Department of Animal Taxonomy and Ecology, Faculty of Biology, Adam Mickiewicz University in Poznań, Uniwersytetu Poznańskieiego 6, 61-614 Poznań (Poland). Ten neoparatypes (slides GR22/14 and GR2/16–2/18) are deposited at the Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Sławkowska 17, 31-016 Kraków (Poland).
Table 1. Measurements [in µm] of selected morphological structures of individuals of *Minibiotus intermedius* 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 (Buccopharyngeal tube) | N  | RANGE | MEAN  | SD  | Neotype |
|---------------------------------|----|-------|-------|-----|---------|
| Body length                     | 20 | 149–245 | 204–29 | 210–220 |         |
| Buccal tube length              | 20 | 17.5–24.3 | 21.7–20.0 | 22.4–19.5 |         |
| Buccal tube external width      | 20 | 1.5–2.7  | 1.9–0.8  | 1.9–0.9 | Neotype |
| Buccal tube internal width      | 20 | 0.4–0.6  | 0.6–2.5  | 0.6–2.7 | Neotype |
| Ventral lamina length           | 19 | 6.2–9.2  | 8.0–37.0 | 7.9–35.3 |         |

| CHARACTER (Placoid lengths)     | N  | RANGE | MEAN  | SD  | Neotype |
|---------------------------------|----|-------|-------|-----|---------|
| Macroplacoid 1                  | 20 | 1.6–2.0 | 1.9–0.8 | 1.9–0.5 | Neotype |
| Macroplacoid 2                  | 20 | 1.3–1.9  | 1.6–7.5  | 1.7–7.6 | Neotype |
| Macroplacoid 3                  | 20 | 1.4–1.9  | 1.7–7.8  | 1.8–8.0 | Neotype |
| Microplacoid                    | 20 | 0.4–0.7  | 0.6–2.6  | 0.6–2.7 | Neotype |
| Macroplacoid row                | 20 | 5.2–7.2  | 6.3–29.1 | 6.5–29.0 | Neotype |

| CHARACTER (Claw I heights)      | N  | RANGE | MEAN  | SD  | Neotype |
|---------------------------------|----|-------|-------|-----|---------|
| External primary branch         | 18 | 4.3–5.8 | 5.2–23.9 | 5.3–23.7 | Neotype |
| External secondary branch       | 16 | 2.8–4.8  | 4.0–18.2 | 3.9–17.4 | Neotype |
| Internal primary branch         | 18 | 4.2–5.6  | 5.0–22.8 | 5.1–22.8 | Neotype |
| Internal secondary branch       | 15 | 2.7–4.5  | 3.8–17.2 | 4.1–18.3 | Neotype |

| CHARACTER (Claw II heights)     | N  | RANGE | MEAN  | SD  | Neotype |
|---------------------------------|----|-------|-------|-----|---------|
| External primary branch         | 17 | 4.5–6.4  | 5.5–25.8 | 5.7–25.4 | Neotype |
| External secondary branch       | 17 | 3.0–5.2  | 4.2–19.6 | 4.8–21.4 | Neotype |
| Internal primary branch         | 17 | 4.1–5.9  | 5.2–24.0 | 5.5–24.6 | Neotype |
| Internal secondary branch       | 17 | 2.9–4.7  | 4.0–18.5 | 4.3–19.2 | Neotype |

| CHARACTER (Claw III heights)    | N  | RANGE | MEAN  | SD  | Neotype |
|---------------------------------|----|-------|-------|-----|---------|
| External primary branch         | 18 | 4.5–6.4  | 5.5–25.7 | 5.6–25.0 | Neotype |
| External secondary branch       | 16 | 3.0–5.5  | 4.3–19.8 | 4.6–20.5 | Neotype |
| Internal primary branch         | 18 | 4.1–6.2  | 5.3–24.1 | 5.0–22.3 | Neotype |
| Internal secondary branch       | 16 | 3.1–5.1  | 4.1–18.7 | 4.0–17.9 | Neotype |

| CHARACTER (Claw IV heights)     | N  | RANGE | MEAN  | SD  | Neotype |
|---------------------------------|----|-------|-------|-----|---------|
| Anterior primary branch         | 13 | 5.3–6.7  | 6.0–26.9 | 6.3–28.1 | Neotype |
| Anterior secondary branch       | 13 | 4.0–5.5  | 4.8–21.3 | 4.4–19.6 | Neotype |
| Posterior primary branch        | 14 | 5.8–6.8  | 6.3–28.1 | 6.5–29.0 | Neotype |
| Posterior secondary branch      | 14 | 4.3–6.0  | 5.0–22.2 | 4.9–21.9 | Neotype |

Table 2. Measurements [in µm] of selected morphological structures of the eggs of *Minibiotus intermedius* 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 (Egg structure)        | N  | RANGE | MEAN  | SD  |
|---------------------------------|----|-------|-------|-----|
| Egg bare diameter               | 8  | 46.3–54.5 | 51.4  | 2.5 |
| Egg full diameter               | 8  | 52.1–61.4 | 58.0  | 2.8 |
| Process height                  | 24 | 3.0–3.9  | 3.4  | 0.2 |
| Process base width              | 24 | 1.3–2.0  | 1.6  | 0.2 |
| Process base/height ratio       | 24 | 41–61%   | 47%  | 5%  |
| Top of processes width          | 24 | 2.4–3.5  | 2.9  | 0.3 |
| Inter-process distance          | 24 | 2.4–4.0  | 3.3  | 0.5 |
| Number of processes on the egg  | 8  | 28–32    | 30.0  | 1.3 |
Figure 1. *Minibiotus intermedius*: adult specimen, dorso-ventral projection (neotype, PCM). Scale bar in µm.

Figure 2. *Minibiotus intermedius*: (A) bucco-pharyngeal apparatus, general view (neoparatype) (filled arrowhead indicates a thickening below stylet insertion point; (B) lateral rows of placoids (neoparatype); (C) central row of placoids (neoparatype). All in PCM. Scale bars in µm.
3.1.1. Short Diagnosis

The body cuticle was smooth without pores and granulation, but very poorly visible granulation on the IV pair of legs was present. Large eye spots were also present. The buccal tube with an anterior and a posterior bend and slightly thicker below the stylet insertion point. The pharyngeal bulb had apophyses, three granular macroplacoids, and a small microplacoid (length sequence $2 \leq 3 < 1$). Eggs with processes (28–32 around the circumference) in the shape of a screw’s head were covered by a separate membrane.
Figure 4. Minibiotus intermedius: (A) egg chorion (PCM); (B) embryo in the egg (PCM); (C, D) egg chorion (SEM). Scale bars in μm.

Figure 5. Minibiotus intermedius: (A) egg surface with processes visible (PCM); (B, C) egg surface with processes (SEM) (filled arrowheads indicate striae); (D) egg processes, lateral view (PCM); (E) egg process (SEM). Scale bars in μm.

3.1.2. Description of the Neotypic Population

Animals (measurements and statistics in Table 1). Body white in live specimens and transparent after fixation in Hoyer’s medium (Figure 1). Eyes were present in all specimens after mounting in the Hoyer’s medium (Figure 1). The body cuticle was smooth without pores and granulation (Figure 1).

Mouth antero-ventral surrounded by ten peribuccal papulae or shortened lamellae. The bucco-pharyngeal apparatus of the Minibiotus type (Figure 2A) had an anterior and a posterior bend (clearly visible in lateral view). The buccal tube was slightly thicker below the stylet insertion point (Figure 2A, arrow). Under PCM, only a third band of teeth was faintly visible (Figure 2A).

The pharyngeal bulb was spherical, with large triangular apophyses, three granular macroplacoids, and very small granular microplacoid placed very close to the third macroplacoid (Figure 2A,B). The macroplacoid length sequence was (2 < 3 < 1). The first macroplacoid narrowed anteriorly (Figure 2A,B). All macroplacoids without constrictions (Figure 2A–C).

Claws stout, of the hufelandi type (Figure 3A,B). The primary branches had very large and distinct accessory points, a common tract, and an evident stalk (Figure 3A,B). Under PCM, very poorly visible granulation was present on the IV pair of legs (visible mainly in larger specimens) (Figure 3C, filled arrowhead). The lunulae was smooth on all legs. The cuticular bars under the claws were absent. Double muscle attachments were faintly marked under PCM (Figure 3A, empty arrowhead).

Eggs (measurements and statistics in Table 2) laid freely, white, spherical, or slightly ovoid (Figure 4A–D). Processes were nail-shaped (shaped like the head of a screw) (Figures 4A–D and 5A–E). Each process was covered by a separate membrane (Figure 5A,B,D). The heads of the processes were always wider than the process bases. In SEM, a central and deep depression was present at the top of the processes (Figure 5B,C,E). This structure was also visible under PCM as a lighter circles at the processes tops (5D). Under PCM the egg surface between process smooth whereas under SEM usually striae extend from each process connecting it with the neighboring processes (Figure 5B,C, arrows). These striae (which are in fact formed by membrane) formed four quadratic areas...
that surrounded each egg process resembling poorly marked areolae. Within each of these areas’ wrinkles form 1–2 flat rose-like whorl structures (Figure 5B,C, arrows).

DNA sequences
We obtained good-quality sequences for the applied molecular markers:
1. COI—GenBank: ON005160, 634 bp long;
2. 18S rRNA—GenBank: ON005188-89, 1182 bp long;
3. 28S rRNA—GenBank: ON005193-95, 753 bp long.

3.2. Comparisons with Other Genetic Sequences of Minibiotus Taxa
All the obtained sequences of M. intermedius were unique and distinct from those deposited in GenBank. The COI molecular marker exhibited a single sequence. In the conservative 18S rRNA gene fragment, we observed no differences between two sequences. In 28S rRNA, two haplotypes were found (three sequences), with a $p$-distance of 0.6%.

The ranges of uncorrected genetic $p$-distances between M. intermedius and other species/taxa belonging to the genus Minibiotus are as follows (please see SM2):
(a) COI: 21.3–28.4% (23.7% on average), with the most similar being M. gumersindoi (FJ435803 [14]), M. furcatus (JX683828-29 [65]), and Minibiotus sp. (MW306857 [66]), and the least similar being M. ioculator (MT023412 [10]);
(b) 18S rRNA: 0.4–1.0% (0.9% on average), with the most similar being M. gumersindoi (FJ435748 [15]), and the least similar being Minibiotus sp. (EU266934 [47]);
(c) 28S rRNA: 4.8–13.7% (9.4% on average), with the most similar being M. gumersindoi (FJ435761 [15]), and the least similar being M. pentannulatus (MT024043 [10]).

3.3. Morphological Differential Diagnosis
Minibiotus intermedius, by the morphology of adults (smooth dorsal and ventral cuticle and absence of pores) and eggs (processes in shape of a screw’s head and covered by a separate membrane), is most similar to: M. continuus (Pilato and Lisi [67]), M. floriparus (Claxton [10]), and M. taiti (Claxton [10]). However it differs from:
1. M. continuus by: the presence of eyes, a different macroplocoid length sequence ($2 \leq 3 < 1$ in M. intermedius vs. $1 = 2 = 3$ in M. continuus), and a higher number of processes on the egg circumference (28–32 in M. intermedius vs. 21–22 in M. continuus).
2. M. floriparus by: a different macroplocoid length sequence ($2 \leq 3 < 1$ in M. intermedius vs. $2 < 1 = 3$ in M. floriparus), a lower $pt$ of the stylet support insertion point ($pt$: 53.8–56.3 in M. intermedius vs. $pt$: ca. 64.4 in M. floriparus), the absence of granulation on legs I–III, the lack of pores on the distal tops of egg processes, larger eggs (egg bare diameter: 46.3–54.5 µm and full diameter: 52.1–61.4 µm in M. intermedius vs. ca. 62.0 µm and ca. 70.0 µm, respectively, in M. floriparus), lower egg processes (3.0–3.9 µm in M. intermedius vs. 5.5–6.0 µm in M. floriparus), narrower tops of processes (2.4–3.5 µm in M. intermedius vs. 6.0–7.0 µm in M. floriparus), and a higher number of processes on the egg circumference (28–32 in M. intermedius vs. 20–22 in M. floriparus).
3. M. taiti by: lower $pt$ of stylet support insertion point ($pt$: 53.8–56.3 in M. intermedius vs. $pt$: ca. 60.3 in M. taiti), the absence of granulation on legs I–III, and the lack of rings of small circles around the central pore on the top of egg processes.

3.4. Establishing of the New Neotype and Neoparatypes of M. intermedius
Taking into consideration that M. intermedius was described in 1888 by Plate, based on specimens from Chile and Germany, we can probably assume that the type material of M. intermedius no longer exists. What is more accurate diagnose of the species were poorly described in the past so that it was necessary to establish a neotype series of this species. Claxton [9] redescribed M. intermedius and established neotype altogether with three syntypes for four specimens collected in Marburg on 27 August 1994. Importantly, Claxton did not report any eggs from this locality, and she unjustifiably assigned eggs from
other localities (Africa, Australia, Europe, New Zealand, and North and South America) for this redescription. This action should be criticized as it is commonly known that cryptic species or complexes of extremely similar species are often reported for tardigrades. In such a case, one should be extremely careful while assigning morphotype of eggs to the animal morphotype, especially in groups such as macrobiotids, in which egg ornaments hold a number of characters which are important in species identification. Moreover, the exact location (with geographic coordinates) in Marburg was also not reported by Claxton [9]. In addition, so far, DNA barcodes are unknown for *M. intermedius*, as confident species identification is currently impossible.

For this reason, considering all issues regarding the Claxton’s neotype, we decided that existing neotype and syntypes are invalid due to the lack of eggs from the locality, which makes the correct identification of the species impossible (see ICZN, article 75.3.4 and 75.3.5). In such a situation, we designate a new neotype (specimen) altogether with 45 neoparatypes (37 specimens and 8 eggs) of *M. intermedius* collected from the type locality in Marburg (Germany), which is in agreement with ICZN article 75.3.5. The detailed characterization of the neotype population by integrated analysis can stabilize the taxonomy of the genus and allow for more detailed exploration of its diversity. Specimens of the neotype series were deposited in the Department of Animal Taxonomy and Ecology of Adam Mickiewicz University in Poznań, Poland, as well as in the Institute of Systematics and Evolution of Animals of the Polish Academy of Sciences, Kraków, Poland. All the above-mentioned statements are in accordance with the International Commission on Zoological Nomenclature (ICZN) acts dedicated to establishing a neotype series.

4. Discussion

In our study, we used integrative taxonomy to describe *M. intermedius* specimens from one of reported terra typica—Marburg (Germany). Our analysis has shown that our specimens and their eggs are morphologically very similar to specimens and eggs used for the first redescription of this species by Claxton [9]. However, in the population studied by us, eggs were a little smaller than those described by Claxton [9], i.e. egg bare diameter: 40.0–45.0 µm (in [9]) vs. 46.3–54.5 µm (in studied population), egg full diameter: 45.0–52.0 µm vs. 52.1–61.4 µm. Moreover, a ring of tiny pores on the top of egg processes was reported by Claxton [9], though these pores were absent in the population in our study. However, as noted previously, eggs described by Claxton [9] were not collected in type locality in Marburg, but in many different localities. Therefore, in this situation, they cannot be confidently assigned to the true *M. intermedius* and it is very likely that they may belong to a completely different *Minibiotus* species. The detailed redescription presented by us in this study with detailed phenotypic and genetic data for the neotype population will effectively prevent similar misunderstandings in the future. This will directly contribute to the knowledge of the true distribution range of this species, which are most probably overestimated at present due to the mentioned identification problem [68–74].

Molecular markers (i.e., COI, 18S rRNA, and 28S rRNA sequences) were only available for four *Minibiotus* species and seven taxa belonging to an undefined species of the genus *Minibiotus*. The ML phylogenetic reconstructions based on the limited molecular data sets yielded a congruent topology (Figure 6) with two main clades: the first clade comprised *M. intermedius* (without pores) and *M. gumersindoi* (with pores) and the second clade contained *M. furcatus* (with pores). For *M. pentannulatus* (with pores) and *M. loculator* (without pores), only COI and 28S rRNA sequences were available which were clustered together with the second clade. It is obvious that the taxonomic position of some sequences deposited in GenBank, described as *M. furcatus*, still needs further verification. We are not convinced which of these sequences have been correctly flagged, and all of them need revision. However, we assumed that the two COI sequences, i.e., deposited in GenBank as JX683828 and JX683829, seem to be questionable because they were clustered with the first clade.
Figure 6. The phylogenetic position (maximum likelihood analyses) of *M. intermedius* (marked in red) generated as follows: COI datasets under the HKY + G model, 18S rRNA datasets under the K2 model, and 28S rRNA datasets under the K2 + G model. The tree with the highest log likelihood is shown. Supporting bootstrap values are given above the branches (nodes with bootstrap <70 were collapsed) and the number of substitution events per site is given below the branches. The GenBank accession numbers of all the sequences applied are given in SM02. Species with a questionable position on the phylogenetic tree were marked in gray stars.
In summary, we propose a new population of the *M. intermedius* from Marburg as a type population of this species. Re-establishing the type species of the genus Minibiotus with provided integrative description should facilitate the description of new taxa within this genus.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14050356/s1, SM1: Raw morphometric data of *Minibiotus intermedius*; SM2: Detailed *p*-distance between analyzed taxa.

**Author Contributions:** Conceptualization, Ł.K.; methodology, Ł.K., M.R., M.G., P.K. and M.M.; validation, Ł.K., M.R., M.G., P.K. and M.M.; formal analysis, Ł.K., M.R., M.G., P.K. and M.M.; investigation, Ł.K., M.R., P.K. and M.M.; resources, Ł.K.; writing—original draft preparation, Ł.K., M.R., M.G., M.P.K. and M.M.; All authors have read and agreed to the published version of the manuscript.

**Funding:** Milena Roszkowska and Pushpalata Kayastha are scholarship passport holders of the future interdisciplinary doctoral studies at the Faculty of Biology, Adam Mickiewicz University, Poznań POWR.03.02.00-00-I006/17. The work of Pushpalata Kayastha was also supported by grant UNIVERSYTET JUTRA No. POWR.03.05.00-00-Z303/17. The work of Monika Mioduchowska was supported by grant no. 2021/43/D/NZ8/00344.

**Institutional Review Board Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Acknowledgments:** Studies have been partially conducted in the framework of activities of the Biodiversity and Astrobiology Research group (BARG).

**Conflicts of Interest:** The authors declare no conflict of interest.

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