

## Research article

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# Integrative description of *Paramacrobotus bengalensis* sp. nov. (Tardigrada: Eutardigrada: Macrobiotidae), a new limno-terrestrial tardigrade species from the state of West Bengal, India

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**Abstract.** *Paramacrobotus bengalensis* sp. nov. was discovered in a moss sample collected from a tree in West Bengal, India. We describe this new species using detailed morphological and morphometric data obtained from phase contrast microscopy and scanning electron microscopy, along with molecular and phylogenetic data analyses. Due to the presence of a cap-like structure at the distal portion of egg processes, the new species showed the highest similarity with *Paramacrobotus garynahi* (Kaczmarek, Michalczyk & Diduszko, 2005), *Paramacrobotus alekseevi* (Tumanov, 2005), *Paramacrobotus filipi* Dudziak, Stec & Michalczyk, 2020, *Paramacrobotus sagani* Daza, Caicedo, Lisi & Quiroga, 2017, *Paramacrobotus vanescens* (Pilato, Binda & Catanzaro, 1991) and *Paramacrobotus gadabouti* Kayastha, Stec, Mioduchowska & Kaczmarek, 2023. However, it can be differentiated from them by some morphological and morphometric characteristics. The genetic data corroborated our phenotypic outcome further supporting the new species hypothesis.

**Keywords.** *Paramacrobotus richtersi* morphogroup, morphometry, new species, COI, 18S rRNA.

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## Introduction

The Phylum Tardigrada Doyère, 1840 includes minute (maximum up to 1.2 mm in length), segmented, eight-legged invertebrates (Campbell *et al.* 2011) that dwell in a variety of terrestrial and aquatic

environments and in all climatic zones (Nelson *et al.* 2015). They comprise more than 1400 species belonging to 159 genera and 33 families (Degma & Guidetti 2009–2023). The family Macrobiotidae Thulin, 1928 consists of eutardigrades categorized by high species diversity and characterized by: i) presence of condensed epicuticle layer that lacks pillar-like structures; ii) presence of double Y-shaped claws with a configuration of 2112 on each leg; iii) presence of ventral lamina on the ventral side of the buccal tube; iv) absence of cephalic papillae; v) free-laid ornamented eggs (Bertolani *et al.* 1996; Guidetti *et al.* 2000; Pilato & Binda 2010; Marley *et al.* 2011). The limno-terrestrial genus *Paramacrobotus* was erected by Guidetti *et al.* (2009) based on molecular and morphological data from the two informal species groups recognized previously within the genus *Macrobiotus* C.A.S. Schultze, 1834 (Guidetti *et al.* 2009). One of them was *Paramacrobotus richtersi* morphogroup which is characterized by: i) animals equipped with three rod-shaped macroplacoids; ii) presence of the microplacoid; iii) areolated eggs with cone-shaped processes (Kaczmarek *et al.* 2017). The second group (*Paramacrobotus areolatus* morphogroup) differs from the *richtersi* morphogroup by the complete absence of microplacoid in the pharynx. Currently, the entire cosmopolitan genus *Paramacrobotus* comprises 45 species.

Tardigrade research in India is very limited, as sparse studies have been conducted since the 1900s. Among the marine tardigrades from the class Heterotardigrada Marcus, 1927, *Stygarctus bradypus* Schulz, 1951 has been reported in Andhra Pradesh (Rao & Ganapati 1968), Odisha (Rao 1969, 1971), and the Andaman and Nicobar Islands (Rao 1975); *Stygarctus lambertii* Grimaldi de Zio, D'Addabbo Gallo, Morone De Lucia & Daddabbo, 1987 has been reported from the Indian Ocean (Grimaldi de Zio *et al.* 1987), whereas Vishnudattan *et al.* (2021) discovered a new species, *Stygarctus keralensis* Vishnudattan, Bijoy Nandan, Hansen & Jayachandran, 2021, from Kerala. Among terrestrial heterotardigrades, *Nebularmis indicus* Gąsiorek, Ciosek & Michalczyk, 2021 was recently discovered in Goa (Gąsiorek *et al.* 2021). From the class Eutardigrada Richters, 1926, Tumanov (2006) reported and described *Milnesium longiungue* Tumanov, 2006 from Ladakh. *Pseudobiotus kathmanae* Nelson, Marley & Bertolani, 1999 have also been reported by Tumanov (2018) from Himachal Pradesh. Coughlan & Stec (2019) described a new species, *Macrobiotus kamilae* Coughlan & Stec, 2019, belonging to the *Macrobiotus hufelandi* morphogroup from Mussoorie. Bhakare & Pai (2021) reported several heterotardigrade and eutardigrade genera (*Barbaria* Michalczyk, Gąsiorek, Morek & Stec, 2019, *Cornechiniscus* Maucci & Ramazzotti, 1981, *Dactylobiotus* R.O. Schuster, 1980, *Thulinus* Bertolani, 2003, *Doryphoribius* Pilato, 1969, *Macrobiotus* Schultze, 1834) from the Western Ghats. Regarding the genus *Paramacrobotus*, only one nominal species, *Paramacrobotus chieregoi* (Maucci & Durante Pasa, 1980), has been reported from India by Maucci & Durante Pasa (1980) from the Andaman Islands and by Suma *et al.* (2020) from Bangalore. Recently, a general consensus among the scientific communities regarding tardigrades has increased, resulting in the advancement of research on this challenging animal group. In this study, we provide an integrative description of a new limno-terrestrial tardigrade species *Paramacrobotus bengalensis* sp. nov. based on detailed morphological and morphometric data of animals and eggs collected using light and scanning electron microscopy. These data were further substantiated by DNA sequences of two genetic markers that are standard in modern tardigrade taxonomy (nuclear 18S rRNA and mitochondrial COI).

## Material and methods

### Sample collection and processing

The samples were collected from 'Acharya Jagadish Chandra Bose Indian Botanic Garden', Shibpur, Howrah, West Bengal, India (Fig. 1). A moss sample was collected from one tree in August 2021. The sample were packed in a paper bag and then dried at 30°C. Tardigrades were extracted and examined using standard methods (Stec *et al.* 2015). The map in Fig. 1 was made in Ocean Data Viewer ver. 5.4.0 (Ocean data view <http://odv.awi.de>).

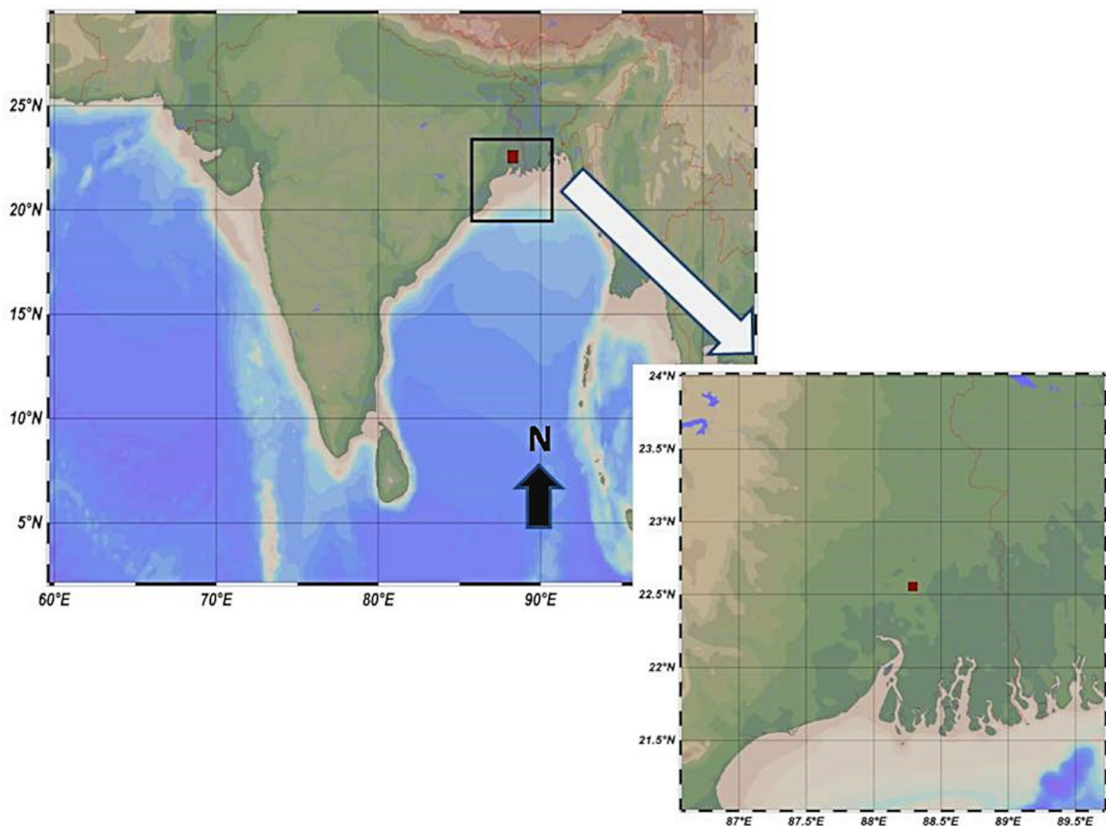
### Microscopy and imaging

Thirty two specimens (one holotype, 28 paratypes, three voucher specimens) and five eggs were mounted on microscope slides in a small drop of Hoyer's medium and secured with coverslips following the protocols described in Morek *et al.* (2016). The slides were then placed in an incubator and dried for 5 days at 60°C. The dried slides were sealed with transparent nail polish and examined under a Nikon Eclipse Ni-U Phase Contrast Microscope (PCM) associated with a Nikon DS-Fi3 high-resolution Microscope digital camera (Nikon Corporation, Minato, Tokyo, Japan).

Three specimens and two eggs for Scanning Electron Microscopy (SEM) were prepared according to the protocols described by Stec *et al.* (2015). The specimens were first subjected to ethanol/water series, then acetone ethanol series, then critical point drying with CO<sub>2</sub>, and finally sputter-coated with a thin layer of gold. Specimens were examined under a Carl-Zeiss EVO-18 Special Edition SEM (Jena, Germany) in the Center for Research in Nanoscience and Nanotechnology, University of Calcutta, Kolkata. All figures were assembled in Corel Photo Paint 2017 edition and Paint.net. For deep structures that could not be entirely focused in a single photograph, a series of 2–6 images were taken for ca 0.50 µm and then stacked into a single deep-focus image using Corel Photo Paint 2017.

### Morphometrics and morphological nomenclature

Sample sizes for morphometry were adjusted following the recommendations of Stec *et al.* (2016a). All measurements are presented in micrometres (µm). The structures were measured only when their orientations were suitable. Body length, excluding hind legs, was measured from the anterior extremity to the end of the body. The buccopharyngeal apparatus and claws were classified as described by Pilato &



**Fig. 1.** Type locality of the new taxa (marked with red square).

Binda (2010). The terminology used to describe the oral cavity armature (OCA) follows Michalczyk & Kaczmarek (2003). The macroplacoid length sequence is indicated in accordance with Kaczmarek *et al.* (2014). The measurement of buccal tube length and the level of stylet support insertion point follow Pilato (1981) and Kaczmarek & Michalczyk (2017). The buccal tube width was measured as the external and internal diameters at the level of the stylet support insertion point. The *pt* index is the ratio of the length of a given structure to the length of the buccal tube and is expressed as a percentage (Pilato 1981). The length of each branch of the claws was measured from the base of the claw (excluding the lunula) to the top of the branch, including accessory points. The height of the processes of the eggs was measured from the base of the process to the apical end. The distance between the egg processes was measured as the shortest distance connecting the bases of two close processes. Morphometric data were handled using the ‘Parachela ver. 1.8’ template available from the Tardigrada Register, [www.tardigrada.net/register](http://www.tardigrada.net/register) (Michalczyk & Kaczmarek 2013). The tardigrade taxonomy followed that of Bertolani *et al.* (2014) and Stec *et al.* (2021). Raw measurements are provided in the supplementary material (Supp. File 1).

### Comparative material

The taxonomic key for the genus *Paramacrobotus* published by Kaczmarek *et al.* (2017) was used to identify the new species. Since it did not result in a perfect hit, the original descriptions of all species of the *Paramacrobotus richtersi* morphogroup were used for comparison with the new species. This comparative study allowed us to identify six morphologically similar taxa for the detailed differential diagnosis, *Paramacrobotus alekseevi* (Tumanov, 2005), *Paramacrobotus filipi* Dudziak, Stec & Michalczyk, 2020, *Paramacrobotus gadabouti* Kayastha, Stec, Mioduchowska & Kaczmarek, 2023, *Paramacrobotus garynahi* (Kaczmarek, Michalczyk & Diduszko, 2005), *Paramacrobotus sagani* Daza, Caicedo, Lisi & Quiroga, 2017, and *Paramacrobotus vanescens* (Pilato, Binda & Catanzaro, 1991) were used.

### Genotyping

DNA was extracted from three individual animals following the protocol described by Casquet *et al.* (2012) with modifications described in detail by Stec *et al.* (2020b). Prior to extraction, each specimen was mounted in water and examined under a microscope at higher magnification for identification. We sequenced four DNA fragments with varying mutation rates: the small ribosomal subunit (18S rRNA, nDNA), the large ribosomal subunit (28S rRNA, nDNA), an internal transcribed spacer (ITS-2, nDNA), and the cytochrome oxidase subunit I (COI, mtDNA). Of the four fragments, only two (18S rRNA and COI) were successfully amplified and sequenced using protocols described in detail by Stec *et al.* (2020b). Three exuviae were successfully extracted, mounted with Hoyer’s medium, and submitted as voucher specimens in Protozoology Section, Zoological Survey of India (ZSI). Primers used in this study and their original references are listed in Table 1. All successfully amplified PCR products were sequenced commercially by Barcode Bioscience Pvt., Ltd (Bengaluru, India). Sequences were manually checked, cleaned, and processed in Bioedit ver. 7.2.5 (Hall 1999) and submitted to GenBank.

### Comparative genetic analysis

For molecular comparison, the published sequences of the two genes (18S and COI) for the genus *Paramacrobotus* were downloaded from GenBank (Table 2). The 18S rRNA sequences were aligned using MAFFT ver. 7 (Katoh *et al.* 2002; Katoh & Toh 2008) with the G-INS-I method (thread = 4, threadtb = 5, threadit = 0, reorder, adjustdirection, anysymbol, maxiterate = 1000, retree 1, global pair input). The COI sequences were aligned according to their amino acid sequences (translated using the invertebrate mitochondrial code) using the MUSCLE (Edgar 2004) algorithm in MEGA XI (Tamura *et al.* 2021) with default settings (all gap penalties = 0, max iterations = 8, clustering method = UPGMB, lambda = 24). Alignments were visually inspected and trimmed in MEGA XI. The Uncorrected Pairwise Distance for the 18S marker gene was calculated using MEGA XI (Supp. File 2).



**Table 1.** PCR primers for amplification of the four DNA fragments sequenced in the study.

DNA Fragment	Primer Name	Primer direction	Primer sequence	Primer source
18S rRNA	18S_Tar_1Ff	Forward	AGGCGAAACCGCGAATGGCTC	Stec <i>et al.</i> (2017)
	18S_Tar_1Rr	Reverse	GCCGCAGGCTCCACTCCTGG	
28S rRNA (amplification failed)	28SF0001	Forward	ACCCVCYNAATTTAAGCATAT	Mironov <i>et al.</i> (2012)
	28SR0990	Reverse	CCTTGGTCCGTGTTTCAAGAC	
ITS2 (amplification failed)	ITS3	Forward	GCATCGATGAAGAACGCAGC	White <i>et al.</i> (1990)
	ITS4	Reverse	TCCTCCGCTTATTGATATGC	
COI	LCO1490	Forward	GGTCAACAAATCATAAAGATATTGG	Folmer <i>et al.</i> (1994)
	HCO2198	Reverse	TAAACTTCAGGGTGACCAAAAAATCA	
	HCOoutout	Reverse	GTAAATATATGRTGDGCTC	Prendini <i>et al.</i> (2005)

### Phylogenetic analysis

To verify the phylogenetic position of the new species, a phylogenetic tree was constructed using the concatenated 18S rRNA + COI sequences of the genus *Paramacrobiotus* with the sequences of five *Tenuibiotus* Pilato & Lisi, 2011, as an outgroup (Table 2). The sequences were concatenated using SequenceMatrix (Vaidya *et al.* 2011). We selected the best partitioning scheme and substitution model for the posterior phylogenetic analysis using Partition-Finder ver. 2.1.1 (Lanfear *et al.* 2016) under the Corrected Akaike Information Criterion (AICc) and the greedy algorithm implemented in the software (Lanfear *et al.* 2012). Because the COI gene codes for proteins, we partitioned our alignment of this marker into three data blocks that correspond to three distinct codon locations before partitioning. PartitionFinder recommended the GTR+I model as the best-fit partitioning scheme for 18S marker gene data block, the GTR+I+G model for the first codon positions of COI, and the GTR+G model for the second and third codon position (Supp. File 3). Bayesian inference (BI) marginal posterior probabilities were calculated using MrBayes ver. 3.2 (Ronquist *et al.* 2012). Two independent runs, each of four Metropolis coupled Markov chains Monte Carlo (MCMC) method, were launched for  $1 \times 10^7$  generations, and trees were sampled every 1000 generations. An average standard deviation of split frequencies of  $<0.01$  was used as a guide to ensure that the two independent analyses had converged. The program Tracer ver. 1.7.1 (Rambaut *et al.* 2018) was then used to ensure that Markov chains had reached stationarity and to determine the correct ‘burn-in’ for the analysis, which was the first 25% of the generations. The ESS values were greater than 200 and a consensus tree was obtained after summarizing the resulting topologies and discarding the ‘burn-in.’ Based on the BI consensus tree, clades recovered with a posterior probability (PP) between 0.95 and 1 were considered well supported, those with a PP between 0.90 and 0.94 were considered moderately supported, and those with a lower PP were considered unsupported. Maximum Likelihood (ML) topologies were constructed using IQ-TREE ver. 2.2.0 (Minh *et al.* 2020). Support for internal nodes was measured using 1000 ultrafast bootstrap replicates (Hoang *et al.* 2018). Bootstrap (BS) support values  $\geq 70\%$  in the final tree were regarded as statistically significant. All final consensus trees were visualised and edited in FigTree ver. 1.4.4 available from <http://tree.bio.ed.ac.uk/software/figtree>.

**Table 2** (continued on next page). GenBank accession numbers for sequences of *Paramacrobiotus* Guidetti, Schill, Bertolani, Dandekar & Wolf, 2009 and *Tenuibiotus* Pilato & Lisi, 2011 (outgroup) species used in this study.

Species Name	18S rRNA	COI	Source
<i>Paramacrobiotus</i> aff. <i>richtersi</i> AU	MH664932	MH675999	Stec <i>et al.</i> (2020c)
<i>Paramacrobiotus</i> aff. <i>richtersi</i> BR 1	MH664934	MH676000	Stec <i>et al.</i> (2020c)
<i>Paramacrobiotus</i> aff. <i>richtersi</i> BR 2		MH676001	Stec <i>et al.</i> (2020c)
<i>Paramacrobiotus</i> aff. <i>richtersi</i> BR 3		MH676002	Stec <i>et al.</i> (2020c)
<i>Paramacrobiotus</i> aff. <i>richtersi</i> FR 1	MH664935	MH676003	Stec <i>et al.</i> (2020c)
<i>Paramacrobiotus</i> aff. <i>richtersi</i> FR 2		MH676004	Stec <i>et al.</i> (2020c)
<i>Paramacrobiotus</i> aff. <i>richtersi</i> HU 1	MH664936	MH676005	Stec <i>et al.</i> (2020c)
<i>Paramacrobiotus</i> aff. <i>richtersi</i> HU		MH676006	Stec <i>et al.</i> (2020c)
<i>Paramacrobiotus</i> aff. <i>richtersi</i> MG 1	MH664938	MH676008	Stec <i>et al.</i> (2020c)
<i>Paramacrobiotus</i> aff. <i>richtersi</i> NO	MH664939	MH676009	Stec <i>et al.</i> (2020c)
<i>Paramacrobiotus</i> aff. <i>richtersi</i> NZ	MH664940	MH676010	Stec <i>et al.</i> (2020c)
<i>Paramacrobiotus</i> aff. <i>richtersi</i> PT 1	MH664944	MH676014	Stec <i>et al.</i> (2020c)
<i>Paramacrobiotus</i> aff. <i>richtersi</i> PT 2		MH676015	Stec <i>et al.</i> (2020c)
<i>Paramacrobiotus</i> aff. <i>richtersi</i> TN	MH664945	MH676016	Stec <i>et al.</i> (2020c)
<i>Paramacrobiotus</i> aff. <i>richtersi</i> TZ	MH664933	MH676017	Stec <i>et al.</i> (2020c)
<i>Paramacrobiotus arduus</i> Guidetti <i>et al.</i> , 2019	MK041032	MK041020	Guidetti <i>et al.</i> (2019)
<i>Paramacrobiotus areolatus</i> (Murray, 1907)	MH664931	MH675998	Stec <i>et al.</i> (2020c)
<i>Paramacrobiotus bengalenseis</i> sp.nov.	ON923868	OP531839	This Study
<i>Paramacrobiotus celsus</i> Guidetti <i>et al.</i> , 2019	MK041031	MK041019	Guidetti <i>et al.</i> (2019)
<i>Paramacrobiotus</i> cf. <i>klymenki</i> IT Pilato <i>et al.</i> , 2012	MH664937	MH676007	Stec <i>et al.</i> (2020c)
<i>Paramacrobiotus</i> cf. <i>klymenki</i> PT Pilato <i>et al.</i> , 2012	MH664943	MH676013	Stec <i>et al.</i> (2020c)
<i>Paramacrobiotus depressus</i> Guidetti <i>et al.</i> , 2019	MK041030	MK041015	Guidetti <i>et al.</i> (2019)
<i>Paramacrobiotus experimentalis</i> Kaczmarek <i>et al.</i> , 2020	MN073468	MN097837	Kaczmarek <i>et al.</i> (2020)
<i>Paramacrobiotus fairbanksi</i> PL Schill <i>et al.</i> , 2010	MH664941	MH676011	Stec <i>et al.</i> (2020c)
<i>Paramacrobiotus filipi</i> 1 Dudziak <i>et al.</i> , 2020	MT261913	MT260372	Stec <i>et al.</i> (2020a)
<i>Paramacrobiotus filipi</i> 2 Dudziak <i>et al.</i> , 2020		MT260373	Stec <i>et al.</i> (2020a)
<i>Paramacrobiotus gadabouti</i> Kayastha <i>et al.</i> , 2023	OP394210	OP394113	Kayastha <i>et al.</i> (2023)
<i>Paramacrobiotus lachowskiae</i> Stec <i>et al.</i> , 2018	MF568532	MF568534	Stec <i>et al.</i> (2018)
<i>Paramacrobiotus metropolitanus</i> Sugiura <i>et al.</i> , 2022	LC637243	LC637242	Sugiura <i>et al.</i> (2022)
<i>Paramacrobiotus richtersi</i> (Murray, 1911)	MK041023	MK040994	Guidetti <i>et al.</i> (2019)
<i>Paramacrobiotus richtersi</i> S38 1 (Murray, 1911)	OK663224	OK662995	Vecchi <i>et al.</i> (2022)
<i>Paramacrobiotus spatialis</i> Guidetti <i>et al.</i> , 2019	MK041024	MK040996	Guidetti <i>et al.</i> (2019)
<i>Paramacrobiotus spatialis</i> S107 1 Guidetti <i>et al.</i> , 2019	OK663225	OK662996	Vecchi <i>et al.</i> (2022)
<i>Paramacrobiotus tonolli</i> US (Ramazzotti, 1956)	MH664946	MH676018	Stec <i>et al.</i> (2020c)

**Table 2** (continued). GenBank accession numbers for sequences of *Paramacrobotus* Guidetti, Schill, Bertolani, Dandekar & Wolf, 2009 and *Tenuibiotus* Pilato & Lisi, 2011 (outgroup) species used in this study.

Species Name	18S rRNA	COI	Source
<b>OUTGROUP</b>			
<i>Tenuibiotus cf. ciprianoi</i>	MN888376	MN888328	Stec <i>et al.</i> (2021)
<i>Tenuibiotus danilovi</i> (Tumanov, 2007)	MN888377	MN888329	Stec <i>et al.</i> (2021)
<i>Tenuibiotus tenuiformis</i> (Tumanov, 2007)	MN888378	MN888330	Stec <i>et al.</i> (2021)
<i>Tenuibiotus voronkovi</i> (Tumanov, 2007)	KX810045	KX810042	Zawierucha <i>et al.</i> (2016)
<i>Tenuibiotus zandrae</i> Stec <i>et al.</i> , 2020	MN443040	MN444827	Stec <i>et al.</i> (2020d)

### Species delimitation

We performed a genetic species delimitation analysis using the Multirate Poisson tree process (mPTP) model (Kapli *et al.* 2017) and assembly species by automatic partitioning (ASAP) (Puillandre *et al.* 2021). The COI dataset for these analyses included newly generated sequences for the new species, as well as all COI sequences of the genus *Paramacrobotus* downloaded from GenBank (Supp. File 4). Outgroups were excluded in both cases to avoid bias produced by a distant relationship between the outgroup and ingroup taxa.

The mPTP web server (<https://mptp.h-its.org/#/tree>) was used to model the multirate Poisson tree process. It is an improved PTP method that does not require user-defined parameters as input and computes support values for each clade using MCMC, which can be used to assess the confidence of ML delimitation (Supp. File 5). The BI tree generated from the COI dataset was used for the mPTP analysis. We used PartitionFinder, as stated in the preceding section, setting three separate data blocks for each codon position, to generate the phylogenetic tree required for the analysis. The best models selected by the program were: JC+I, HKY+G, K80+G (Supp. File 6). Then we used MrBayes ver. 3.2 and the identical settings as mentioned in the preceding section, we calculated Bayesian inference (BI) marginal posterior probabilities using the COI alignment. The outgroup was removed from the final BI tree that we used for the mPTP analysis.

ASAP analyses were performed on the corresponding server (<https://bioinfo.mnhn.fr/abi/public/asap/>). Based on pairwise genetic distances, ASAP separates the species. Additionally, ASAP calculates a relative gap width metric (W), the probability of panmixia (p-val), and ranks the findings according to the ASAP score, with a lower score indicating better partitioning. The number of molecular operational taxonomic units (MOTUs) predicted by ASAP 1<sup>st</sup> scores were selected (Supp. File 7). Additionally, the Uncorrected Pairwise distance was calculated using MEGA XI for all available COI sequences of the genus *Paramacrobotus*; the results are provided in Supp. File 8.

## Results

### *Taxonomic account of the new species*

Phylum Tardigrada Doyère, 1840  
Class Eutardigrada Richters, 1926  
Order Parachela Schuster, Nelson, Grigarick & Christenberry, 1980  
Superfamily Macrobiotoida Thulin, 1928 (in Marley *et al.* 2011)  
Family Macrobiotidae Thulin, 1928  
Genus *Paramacrobotus* Guidetti, Schill, Bertolani, Dandekar & Wolf, 2009

#### *Paramacrobotus bengalensis* sp. nov.

[urn:lsid:zoobank.org:act:19ABAA28-D46D-4045-A126-FC628526C114](https://zoobank.org/act:19ABAA28-D46D-4045-A126-FC628526C114)

Figs 2–7, Tables 3–4

#### Etymology

The specific toponymic *bengalensis* refers to the State of West Bengal, India, where the type locality of this new species, ‘Acharya Jagadish Chandra Bose Indian Botanic Garden’, Shibpur, is situated.

#### Material examined

32 specimens (one holotype, 28 paratypes, three voucher specimens) and five eggs were mounted on microscopic slides in Hoyer’s medium. Three specimens and two eggs were fixed for SEM preparation. Three specimens were processed for genotyping.

#### Holotype

INDIA • West Bengal, Howrah, Shibpur, Acharya Jagadish Chandra Bose Indian Botanic Garden (Fig. 1); 22°33′18.1″ N, 88°17′30.7″ E; Rahul Babu and Subhrangshu Basu leg.; moss from a tree (Sample Code: BSI/M 8); ZSI-HQ/GNC/T1/1.

#### Paratypes

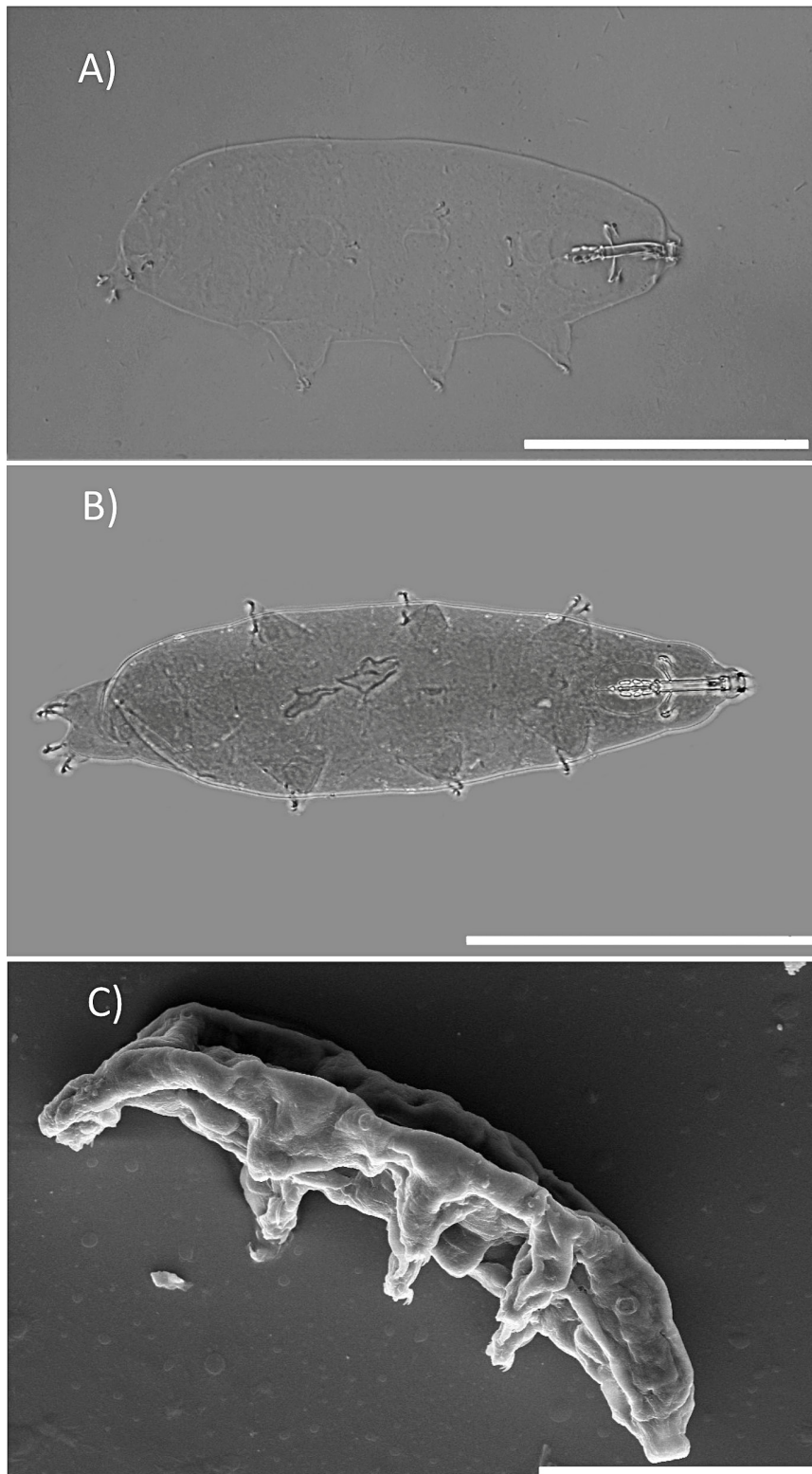
INDIA • 3 specs (slides); same collection data as for holotype; ZSI-HQ/GNC/T2/1, ZSI-HQ/GNC/T3/1, ZSI-HQ/GNC/T4/1 National Zoological Collection, Zoological Survey of India, Kolkata, India • 24 specs (slides); same collection data as for holotype; ZSI/TAR\_IND/WB/001 to ZSI/TAR\_IND/WB/024 National Zoological Collection, Zoological Survey of India, Kolkata, India • 3 voucher specs; same collection data as for holotype; ZSI/TAR\_IND/WB/V001 to ZSI/TAR\_IND/WB/V003 • 5 eggs; same collection data as for holotype; ZSI/TAR\_IND/WB/E001 Protozoology Section, Zoological Survey of India HQ, New Alipore, M Block, Kolkata, India.

#### Description

The body is almost transparent in juveniles, white in adults, and transparent after mounting in Hoyer’s medium (Fig. 2; measurements and statistics provided in Table 3). Eyes are present in live specimens but dissolve after mounting in Hoyer’s medium.

Mouth anteroventral, bucco-pharyngeal apparatus of the *Macrobiotus* type (Fig. 3) with 10 peribuccal lamellae and ventral lamina. The oral cavity armature is well developed and composed of three bands of teeth (Fig. 3B–C). The teeth in the first band are granular in shape and smaller than those in the other two bands. The first band of teeth is situated in the anterior portion of the oral cavity behind the bases of the peribuccal lamellae. The second band, situated between the ring fold and the third band (Fig. 3B–C) is intermediate in size, continuous, and arranged in a row that runs around the oral cavity wall. The second band comprises cone shaped teeth which are parallel to the main axis of the buccal tube. Teeth of the



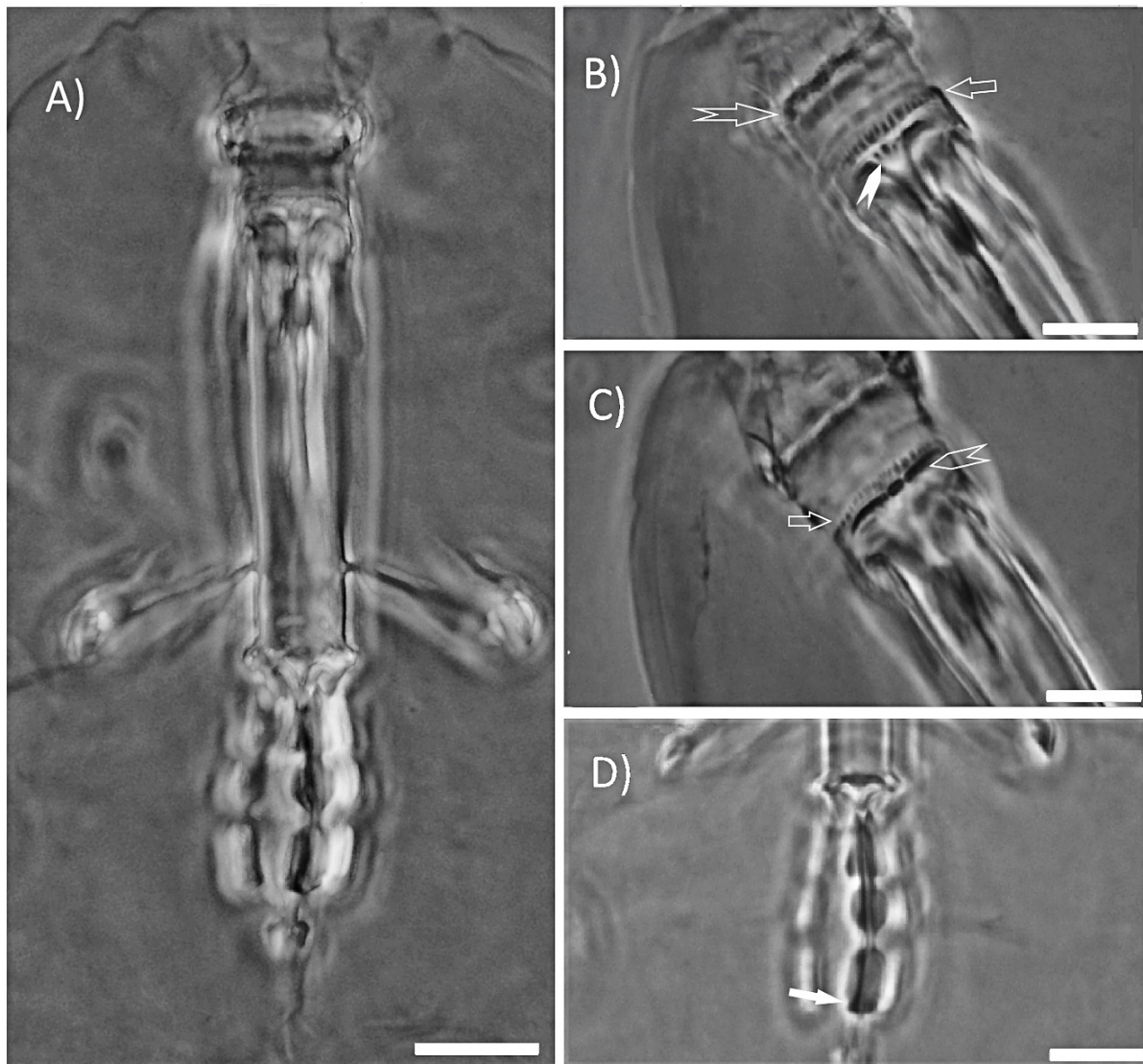


**Fig. 2.** *Paramacrobotus bengalensis* sp. nov. **A.** Holotype (ZSI-HQ/GNC/T1/1), habitus, dorso-ventral projection. **B–C.** Paratypes (ZSI-HQ/GNC/T2/1). **B.** Habitus dorsal projection. **C.** Scanning electron microphotograph of a whole specimen. Scale bars: A, C = 100  $\mu$ m; B = 200  $\mu$ m.

**Table 3.** Measurements (in  $\mu\text{m}$ ) of selected morphological structures of individuals of *Paramacrobiotus bengalensis* sp. nov. mounted in Hoyer’s medium. Abbreviations: 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		Holotype	
		$\mu\text{m}$	<i>pt</i>	$\mu\text{m}$	<i>pt</i>	$\mu\text{m}$	<i>pt</i>	$\mu\text{m}$	<i>pt</i>
Body length	29	158–412	780–1117	295	899	85	94	193	925
Buccal tube									
Buccal tube length	29	19.2–47.0	–	32.8	–	9.2	–	20.9	–
Stylet support insertion point	29	13.5–37.1	70.3–79.6	25.1	76.3	7.3	2.2	16.3	77.9
Buccal tube external width	29	3.6–8.7	17.2–22.1	6.4	19.4	1.8	1.2	3.6	17.2
Buccal tube internal width	29	2.8–7.8	13.1–16.6	5.1	15.4	1.6	1.1	2.8	13.2
Ventral lamina length	29	9.9–25.6	49.2–54.5	17.2	52.4	4.9	1.6	10.6	50.8
Placoid lengths									
Macroplacoid 1	29	2.8–7.1	14.2–15.5	4.8	14.7	1.4	0.4	3.0	14.2
Macroplacoid 2	29	1.9–5.0	9.7–11.8	3.5	10.8	1.0	0.5	2.4	11.3
Macroplacoid 3	29	3.0–7.0	14.1–16.7	5.0	15.5	1.3	0.7	3.3	15.6
Microplacoid	29	1.0–2.7	4.4–7.3	1.8	5.5	0.5	0.7	1.1	5.5
Macroplacoid row	29	7.7–18.2	36.6–42.8	13.3	40.7	3.7	1.4	8.7	41.9
Placoid row	29	10.3–24.5	48.0–55.0	17.0	51.9	4.7	1.6	11.5	55.0
Claw I heights									
External primary branch	26	3.0–7.7	14.9–18.5	5.3	16.1	1.4	0.7	3.9	18.5
External secondary branch	26	2.3–6.0	10.3–13.0	4.1	12.3	1.1	0.6	2.7	12.8
Internal primary branch	25	3.0–7.7	15.2–18.4	5.3	16.2	1.4	0.7	3.9	18.4
Internal secondary branch	24	2.3–6.0	11.0–13.3	4.0	12.3	1.2	0.5	2.8	13.3
Claw II heights									
External primary branch	27	3.1–7.8	15.0–18.4	5.3	16.1	1.4	0.6	3.8	18.4
External secondary branch	27	2.2–6.0	10.2–13.4	4.0	12.2	1.2	0.7	2.5	12.0
Internal primary branch	26	3.2–7.8	14.9–17.6	5.4	16.1	1.4	0.6	3.7	17.6
Internal secondary branch	25	2.3–5.9	10.9–13.0	4.1	12.2	1.1	0.6	2.4	11.4
Claw III heights									
External primary branch	27	3.1–7.7	14.8–18.1	5.3	16.2	1.5	0.6	3.8	18.1
External secondary branch	27	2.2–5.9	10.0–13.6	4.0	12.2	1.2	0.8	2.4	11.4
Internal primary branch	23	3.2–7.2	14.8–17.6	5.3	16.1	1.4	0.6	3.7	17.6
Internal secondary branch	22	2.3–5.4	10.6–13.2	3.9	12.2	1.1	0.6	2.3	11.0
Claw IV heights									
Anterior primary branch	25	4.5–9.7	20.0–25.5	7.3	22.2	1.7	1.3	5.3	25.5
Anterior secondary branch	25	3.4–8.5	16.5–19.5	6.0	18.2	1.6	0.8	3.9	18.4
Posterior primary branch	22	4.7–9.7	20.1–25.6	7.1	22.5	1.8	1.3	5.4	25.6
Posterior secondary branch	20	3.5–8.5	16.8–19.7	5.8	18.3	1.7	0.8	3.9	16.8

second band are uniform and regular and are not joined to each other. The third band is located at the rear end of the oral cavity between the second band teeth and the buccal tube opening. The third band is divided into two parts: dorsal and ventral, with three ventral and three dorsal teeth each (two lateral and one median, which is always slightly shorter than the lateral ones). The dorsal and ventral portions are visible under the PCM as one median ridge and two lateral transverse ridges. The medioventral tooth of the third band of teeth is subdivided into two to three smaller teeth (Fig. 3B). Additional granular teeth are absent between the second and third band of teeth on the ventral side. The pharyngeal bulb is spherically shaped with triangular apophyses. Three macropilacoids and rod-shaped micropilacoids are present and distinctly visible under PCM (Fig. 3A, D). The macropilacoid sequence is  $2 < 1 \leq 3$ , and

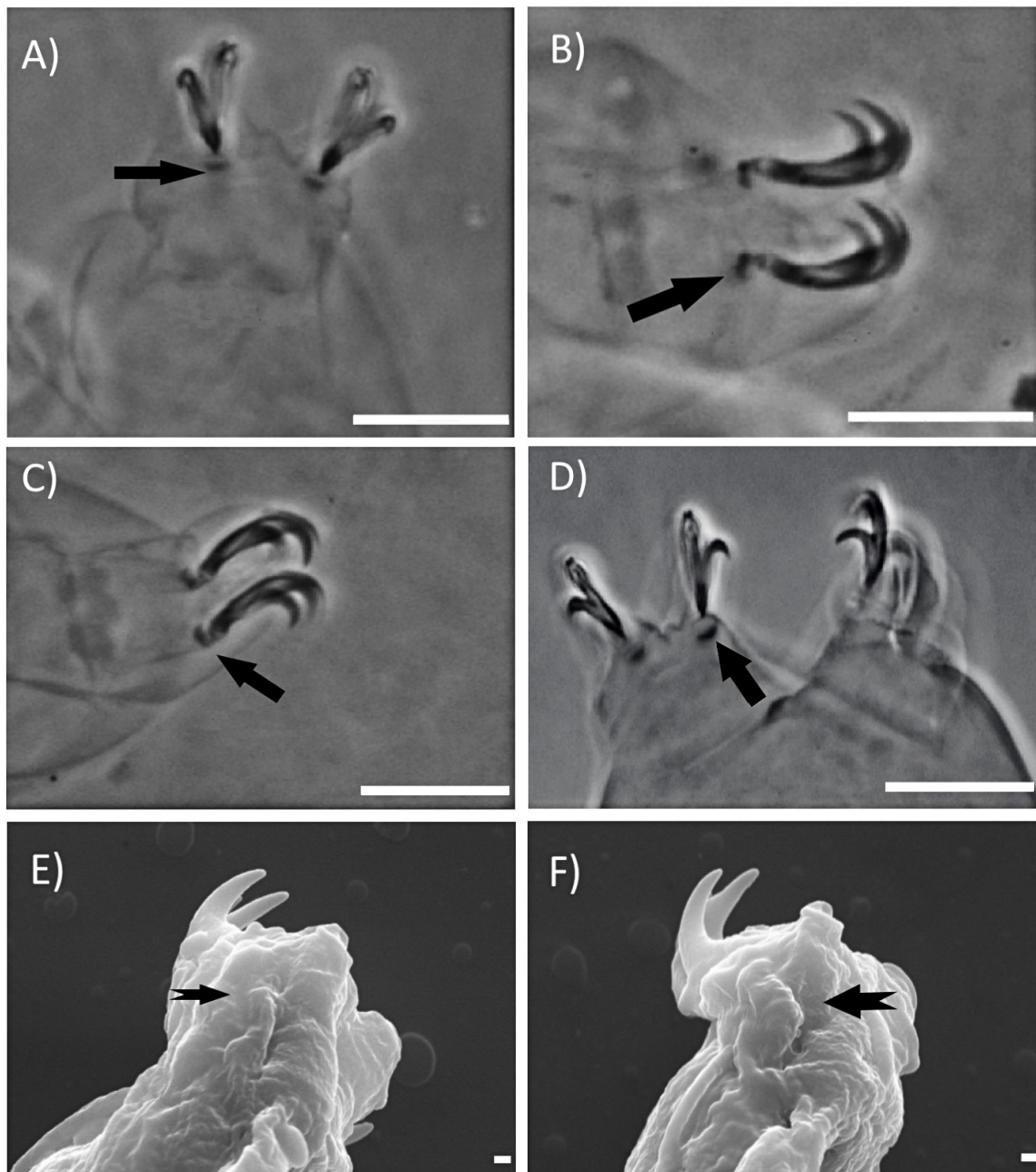


**Fig. 3.** *Paramacrobotus bengalensis* sp. nov., paratypes. **A, D.** Specimen ZSI-HQ/GNC/T3/1. **B–C.** Specimen ZSI/TAR\_IND/WB/024. **A.** Buccal apparatus, dorso-ventral projection with dorsal teeth in focus. **B–C.** Oral cavity armature (B, ventral teeth; C, dorsal teeth). **D.** Macropilacoids. White unfilled arrow indicate the second band of teeth, unfilled chevron arrow indicate the first band of teeth, white unfilled chevron arrow indicate the third band of teeth, white filled chevron arrow indicates medioventral teeth, a white filled arrow indicates the subterminal constriction in the third macropilacoid. All PCM. Scale bars: A = 10 μm; B–D = 5 μm.



the first macroplacoid is anteriorly thinner and arrow-shaped. The second macroplacoid is bar-shaped without constriction, whereas the third macroplacoid has a distinct sub-terminal constriction (Fig. 3D).

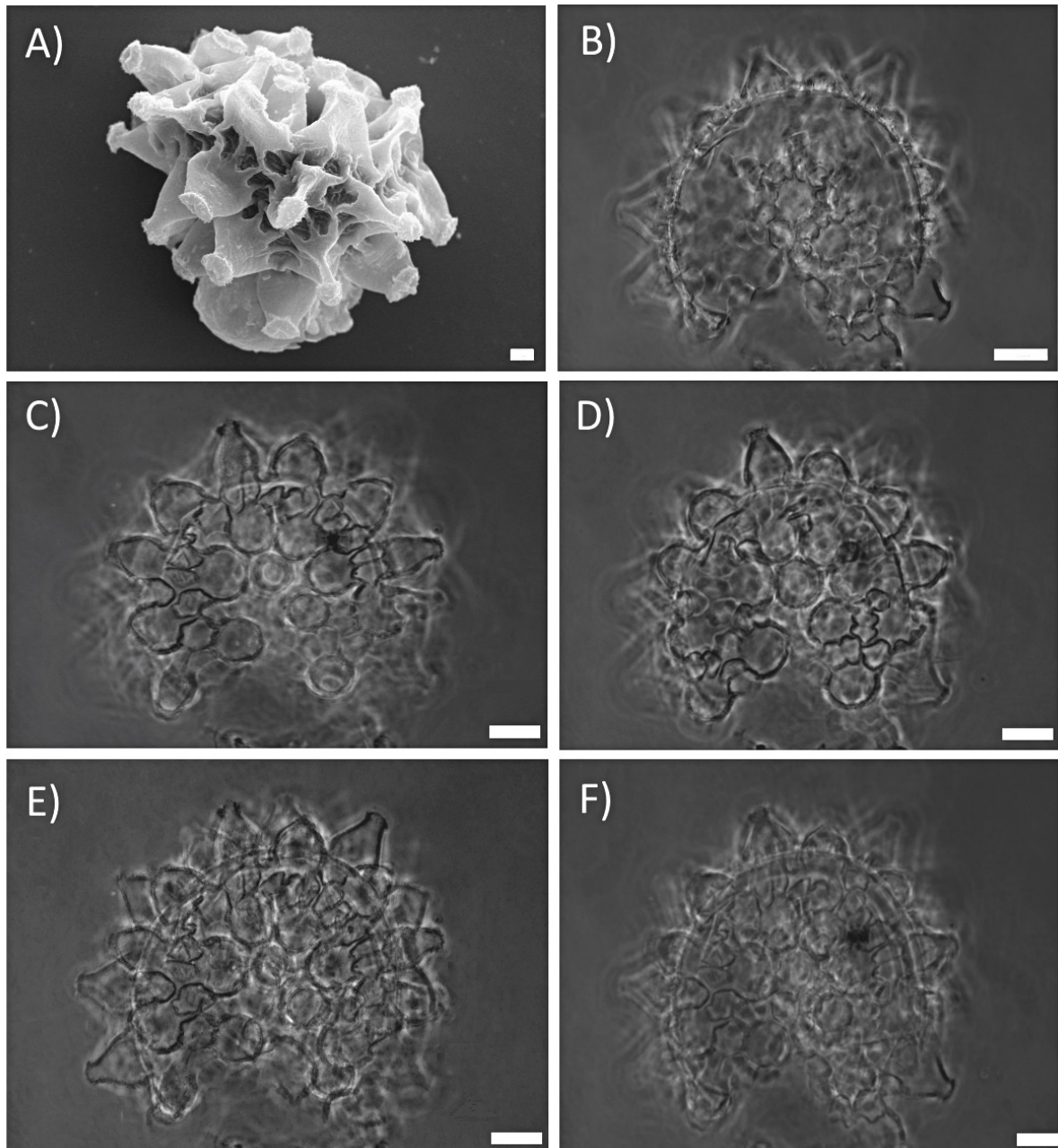
The claws are Y-shaped and of the *hufelandi* type. The primary claw branches have distinct accessory points, a common tract, and a stalk that connects the claw and lunula (Fig. 4A). The lunulae under all



**Fig. 4.** *Paramacrobotus bengalensis* sp. nov., claws. **A.** Holotype (ZSI-HQ/GNC/T1/1), claws I with small smooth lunulae. **B–C.** Paratype (ZSI/TAR\_IND/WB/024). **B.** Claws II with small smooth lunulae. **C.** Claws III with small smooth lunulae. **D.** Paratype (ZSI-HQ/GNC/T2/1), claws IV with smooth lunulae, (black filled arrow indicates smooth lunulae for A–D). **E–F.** Claws II and III without any visible granulation the legs (black filled checkered arrow). Figures assembled from several photos. A–D = PCM; E–F = SEM. Scale bars: A–C = 5  $\mu$ m; D = 10  $\mu$ m; E–F = 0.4  $\mu$ m.

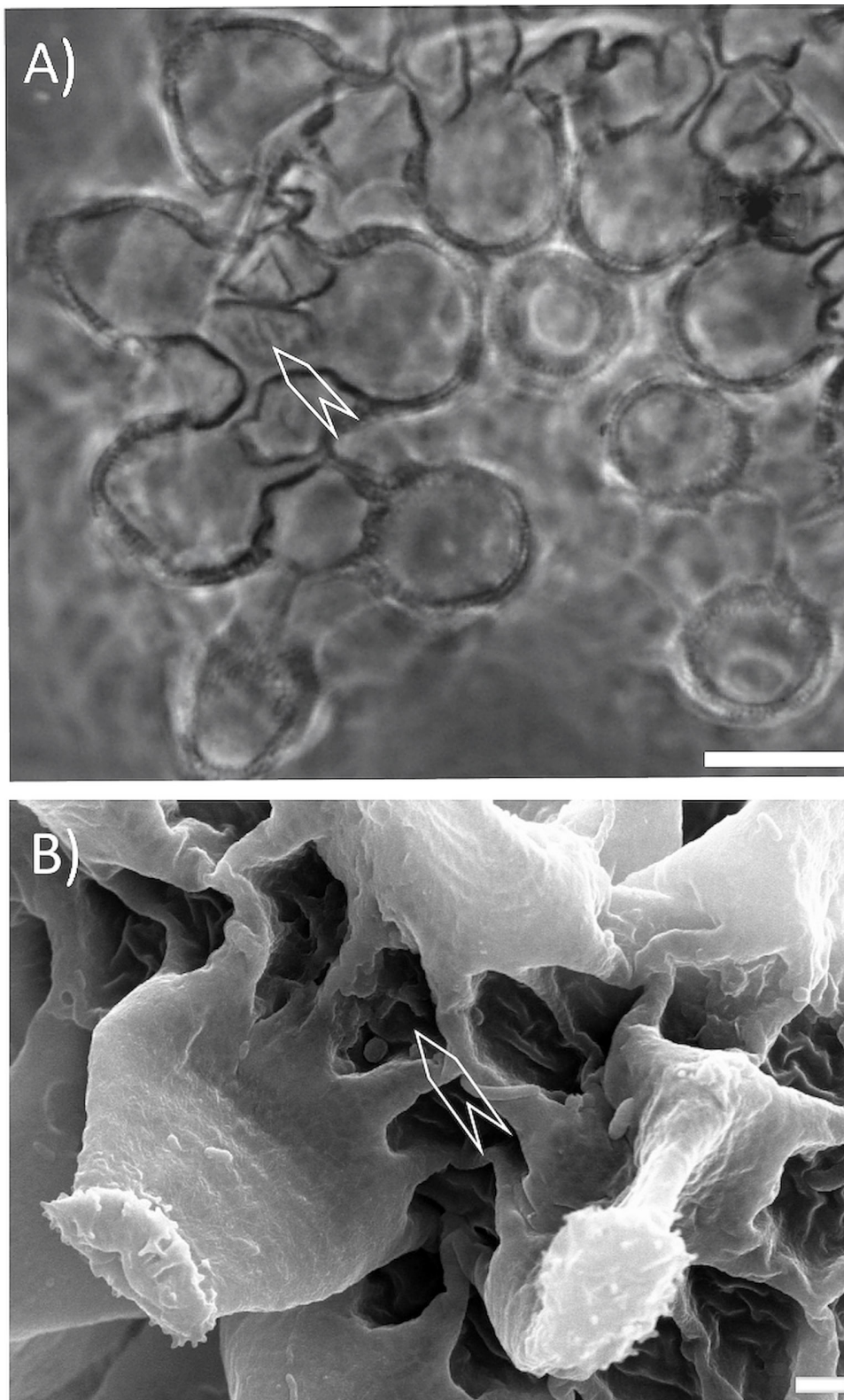
the claws on all the legs are smooth (Fig. 4). Leg cuticle is smooth, without any granulations present in legs I–III. Granulation is present on the hind legs but only faintly visible (Fig. 4D). Cuticular bars under the claws absent. In PCM, muscle attachments under claws I to III are visible (Fig. 4B–C).

Eggs laid freely, white/colourless with 12–14 cone-shaped processes on the circumference (Fig. 5; measurements and statistics provided in Table 4). The space between processes is areolated with 8 to 10 areolas present around each process (Fig. 6). The surface of the areoles is without pores but sculptured with wrinkles. Processes trunk cone shaped with a cap-like structure on the top (Fig. 7), with fine villi-



**Fig. 5.** *Paramacrobotus bengalensis* sp. nov., paratype (ZSI/TAR\_IND/WB/E001). **A.** Entire egg under SEM. **B–F.** Eggs seen in PCM under 1000× magnification. **B.** Midsection. **D–F.** Surfaces. Scale bars: A = 3 μm; B–F = 10 μm.

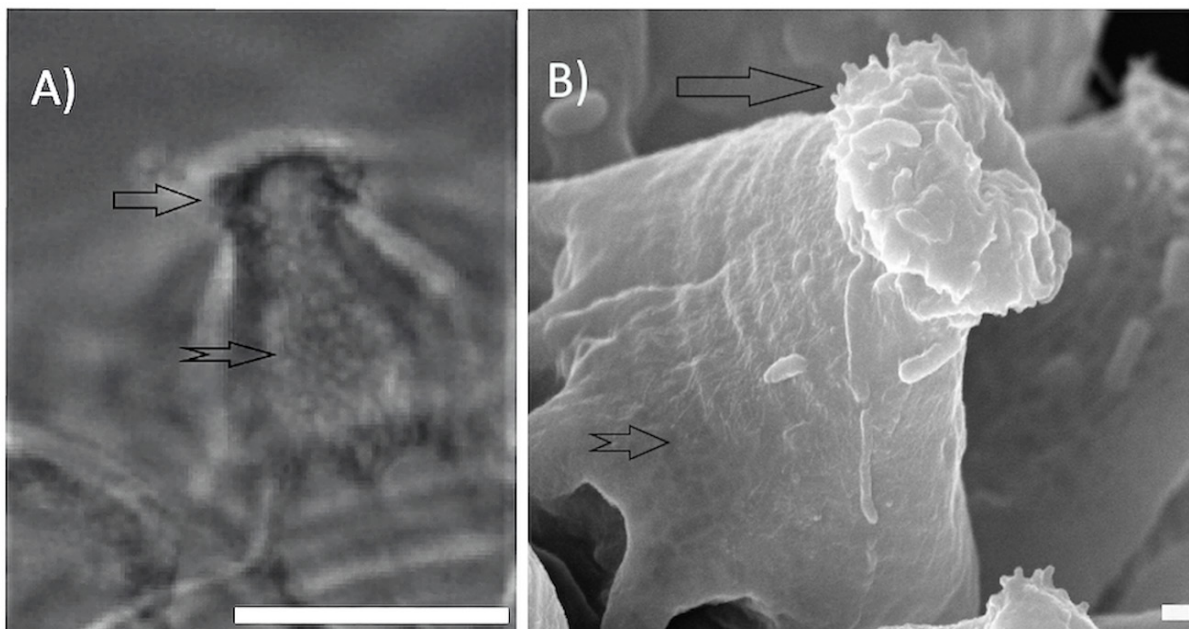




**Fig. 6.** *Paramacrobiotus bengalensis* sp. nov., paratype (ZSI/TAR\_IND/WB/E001), egg chorion details. **A–B.** Zoom on egg surface between the processes with clearly visible areolae and cap like structures on processes (SEM), white empty chevron arrows indicate wrinkles inside the areolae. Scale bars: A = 5  $\mu$ m; B = 2  $\mu$ m.

**Table 4.** Measurements (in  $\mu\text{m}$ ) of selected morphological structures of eggs of *Paramacrobotus bengalensis* sp. nov. mounted in Hoyer's medium. Abbreviations: 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	5	64.6–73.6	68.8	4.0
Egg full diameter	5	87.3–98.5	92.6	4.6
Process height	15	12.4–14.7	13.3	0.8
Process base width	15	9.1–11.1	9.9	0.7
Process base/height ratio	15	70%–78%	75%	2%
Cap Like structure width	15	5.3–7.2	6.1	0.6
Inter-process distance	15	5.3–6.7	6.1	0.4
Number of processes on the egg circumference	5	12–14	13.2	0.8



**Fig. 7.** *Paramacrobotus bengalensis* sp. nov., paratype (ZSI/TAR\_IND/WB/E001), egg process details. **A–B.** An egg process under PCM and SEM with weakly visible reticulum. Black empty arrows indicate the cap like structure on the top of the process, black empty checkered arrows indicate the fine reticulation on the process. Scale bars: A = 10  $\mu\text{m}$ ; B = 1  $\mu\text{m}$ .

like protrusions. Under PCM egg processes walls have fine reticulation which is caused by the internal labyrinthine layer within the chorion (Fig. 7A–B).

### DNA sequences

We obtained sequences for two DNA markers. Out of these two successfully sequenced markers, 18S rRNA was represented by two haplotypes, whereas a single haplotype was found for COI: the 18S rRNA haplotype 1 sequence (GenBank: ON923868), 1017 bp long; the 18S rRNA haplotype 2 sequence (GenBank: ON923866) 1014 bp long; the COI haplotype 1 sequence (GenBank: OP531839), 658 bp long.

### Phylogenetic analysis

The phylogenetic reconstruction performed with the BI and ML methods on the concatenated dataset of the two DNA markers showed almost identical topologies, with lower support values for the ML tree (Fig. 8A). Our analysis revealed that the *Paramacrobotus richtersi* morphogroup forms a monophyletic clade, whereas the *Paramacrobotus areolatus* morphogroup was recovered as a paraphyletic group (Fig. 8A–B), which was consistent with the results presented by Stec *et al.* (2020c). Phylogenetic analysis supported the discovery of *Paramacrobotus bengalensis* sp. nov. The new species is strongly supported in the monophyletic clade of *Paramacrobotus richtersi* in both the BI and ML phylogenetic trees, establishing that it does indeed belong to the *Paramacrobotus richtersi* morphogroup.

### Ecological information

The moss (species unknown) was collected from the mango tree *Mangifera indica* L., at a height of approximately 2 m from the ground. The altitude of the type locality is 12 m above sea level. The type locality is situated on the banks of River Ganges.

### Species delimitation

ASAP and mPTP analyses of 81 COI sequences (Supp. File 4) identified 24 partitioned subsets (asap-score = 6.0) and 30 delimited species. Both analyses revealed *Paramacrobotus bengalensis* sp. nov. as a putative new species.

## Discussion

### Phenotypic differential diagnosis

With the presence of microplacoid *Paramacrobotus bengalensis* sp. nov. is assigned to *Paramacrobotus richtersi* morphogroup. According to Kaczmarek *et al.* (2017) only two species within this group have egg processes terminated with cap-like structures, namely *Paramacrobotus garynahi* (Kaczmarek, Michalczyk & Diduszko, 2005) and *Paramacrobotus alekseevi* (Tumanov, 2005). This structure was also directly reported for recently described species *Paramacrobotus filipi* Dudziak, Stec & Michalczyk, 2020 and *Paramacrobotus gadabouti* Kayastha, Stec, Mioduchowska & Kaczmarek, 2023. Importantly, our literature studies based on original descriptions of taxa belonging to *Paramacrobotus richtersi* morphogroup allowed us to identify two other species of *Paramacrobotus* in which the cap-like structure was not directly reported but seems to be present. These are *Paramacrobotus sagani* Daza, Caicedo, Lisi & Quiroga, 2017 and *Paramacrobotus vanescens* (Pilato, Binda & Catanzaro, 1991). Therefore, we provide below the differential diagnosis that compares our new species with the six taxa mentioned above. The new species can be differentiated from:

*Paramacrobotus alekseevi*, known only from the type locality in Thailand (Tumanov 2005) and from China (Beasley & Miller 2007), by: the medio-ventral tooth usually subdivided into two (only in rare

cases into three) smaller teeth (the medio-ventral tooth always subdivided into three to five smaller teeth in *P. alekseevi*), the absence of granulation in legs I–III (fine granulation present in *P. alekseevi*).

*Paramacrobotus filipi*, reported from the type locality in Malaysia (Stec *et al.* 2020a), by: the absence of granulation on body cuticle (granulation present in *P. filipi*), the absence of granulation in legs I–III (fine granulation present in *P. filipi*), a shorter egg processes (process height: 12.5–14.7  $\mu\text{m}$  in new species vs 17.8–25.2  $\mu\text{m}$ , in *P. filipi*), the absence of pores in egg areoles (porous areoles present in *P. filipi*).

*Paramacrobotus gadabouti*, reported from the type locality in Madeira (Portugal), but also additional localities in Portugal, France, Tunisia and Australia (Kayastha *et al.* 2023), by: the presence of eyespots (eyespots absent in *P. gadabouti*), the absence of granulation in legs I–III (fine granulation present in *P. gadabouti*), the absence of pores in egg areoles (sculptured areoles with pores in *P. gadabouti*).

*Paramacrobotus garynahi*, known only from the type locality in Russia (Kaczmarek *et al.* 2005), by: the presence of eyespots (absent in *P. garynahi*), the absence of granulation in legs I–III (granulation present in *P. garynahi*), smaller eggs (egg bare diameter and full diameter: 64.6–73.6 and 87.3–98.5  $\mu\text{m}$ , in new species vs 96.0–132.0 and 142–180  $\mu\text{m}$  in *P. garynahi*), a shorter egg process (12.4–14.7  $\mu\text{m}$  in new species vs 18.0–30.0  $\mu\text{m}$  in *P. garynahi*) and narrower process bases (9.1–11.1  $\mu\text{m}$  in new species vs 20.0–42.0  $\mu\text{m}$  in *P. garynahi*).

*Paramacrobotus sagani*, reported from the type locality in Colombia (Daza *et al.* 2017), by: the absence of granulation on body cuticle (granulation present in *P. sagani*), the absence of cuticular bars under claws (poorly developed bars present in *P. sagani*), the absence of granulation in legs I–III (fine granulation present in *P. sagani*), the absence of pores in egg areoles (porous areoles present in *P. sagani*), smaller egg bare diameter (64.6–73.6  $\mu\text{m}$  in new species vs 73.7–87.7  $\mu\text{m}$  in *P. sagani*).

*Paramacrobotus vanescens*, reported from the type locality in Tanzania (Pilato *et al.* 1991), by: the presence of eyespots (eyespots absent in *P. vanescens*), an obvious microplacoid in new species (described as, “little, faint, sometimes almost invisible” in *P. vanescens*), the absence of granulation in legs I–III (fine granulation present in *P. vanescens*), the absence of cuticular bars under the claws (faint bars present in *P. vanescens*), a shorter egg processes (12.4–14.7  $\mu\text{m}$  in new species vs 16–17  $\mu\text{m}$  in *P. vanescens*).

### Genotypic differential diagnosis

The ranges of uncorrected genetic p-distances between the new species and species of the genus *Paramacrobotus*, for which sequences are available in GenBank and are as follows (from the most to the least conservative):

18S rRNA: 0.32%–3.1% (1% on average), with the most similar being *Paramacrobotus metropolitanus* Sugiura, Matsumoto & Kuneida, 2022 from Tokyo, Japan (LC637243) and the least similar being *Paramacrobotus areolatus* (Murray, 1907) from Norway (MH664931).

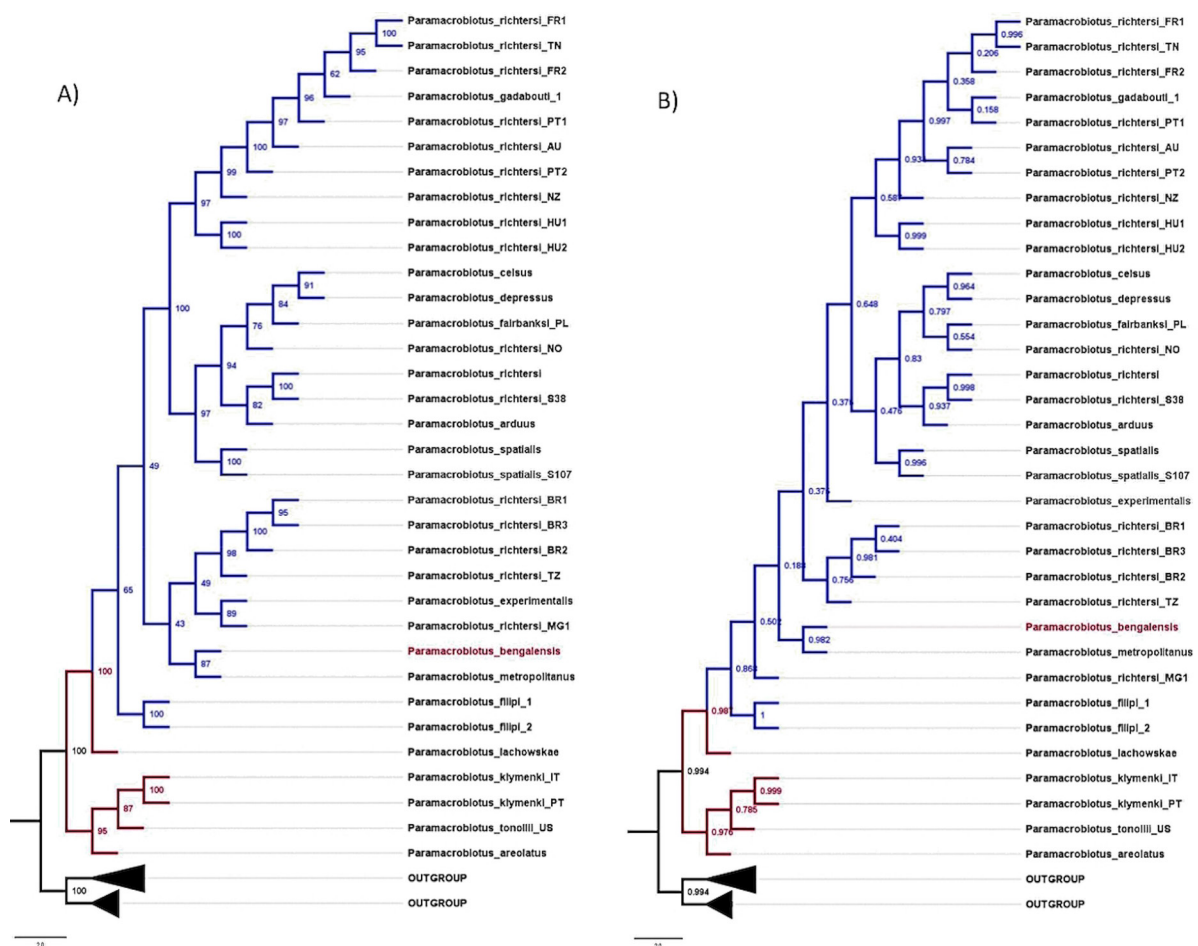
COI: 21%–30% (23% on average), with the most similar being *Paramacrobotus metropolitanus* Sugiura, Matsumoto & Kuneida, 2022 from Tokyo, Japan (LC649796) and the least similar being *Paramacrobotus* sp. *richtersi\_group 3* (EU244599) from Kenya.

### Molecular phylogeny and species delimitation

The fifth phylogeny of the genus *Paramacrobotus* is presented in this study. The first was presented by Guidetti *et al.* (2009), who used two distinct phylogenetic analyses based on 18S rRNA and COI sequences to establish this taxon from the genus *Macrobotus* Schultze, 1834. Guidetti *et al.* (2019) conducted a



second investigation of the genus *Paramacrobilotus*, which included 11 species. The authors re-described the nominal species *Paramacrobilotus richtersi*, as well as several additional new species from this genus. The phylogenetic results obtained by Guidetti *et al.* (2019) were subsequently corroborated by Stec *et al.* (2020c) and Kayastha *et al.* (2023), these studies revealed that the *Paramacrobilotus areolatus* morphogroup is paraphyletic while *Paramacrobilotus richtersi* forms a monophyletic clade using two or four concatenated genetic markers, respectively (18S+COI; 18S+28S+ITS2+COI). This outcome caused the suppression of formerly proposed subgeneric division within *Paramacrobilotus* by Kaczmarek *et al.* (2017). Our study recovered a nearly identical tree topology, which is consistent with the findings of Stec *et al.* (2020c). *Paramacrobilotus bengalensis* sp. nov. cluster with other *Paramacrobilotus richtersi* morphogroup species (Fig. 8A–B). The analysis also indicated that *Paramacrobilotus bengalensis* is closely related to *Paramacrobilotus metropolitanus* Sugiura, Matsumoto & Kuneida, 2022 from Tokyo, Japan. This was also evident when examining the genetic distances that showed a significant level of similarity between DNA sequences of nuclear markers (p-distance; 18S rRNA: 0.32%) and the COI



**Fig. 8.** Phylogeny of *Paramacrobilotus* Guidetti, Schill, Bertolani, Dandekar & Wolf, 2009 based on the nucleotide sequences of two markers (18S rRNA + COI). **A.** ML tree. **B.** BI tree. The numbers at nodes represent Bayesian posterior probability and bootstrap support values. The blue coloured clade represents species from the *Paramacrobilotus richtersi* morphogroup, while the red coloured clade represents species from the *Paramacrobilotus areolatus* morphogroup. Both trees have a red colour for the new species. The outgroup is denoted by black triangles. The scale bar reflects the number of substitutions per position.



dataset, which had the lowest genetic distance among all comparisons with other taxa of *Paramacrobiotus* (p-distance; COI: 21%). Interestingly, although the eggs of our new species and *Paramacrobiotus metropolitanus* are different (the latter lacks the cap-like structure), the genetic similarity and hence close phylogenetic relationship is reflected in the distribution of leg granulation. Both these species have been confirmed to have granulation present only on the hind legs. This may further support the previous suggestions that the evolution of egg morphology is faster/more dynamic than animal morphology (Guidetti *et al.* 2013; Stec *et al.* 2016b; Stec 2022). Finally, both mPTP and ASAP delineation based on COI sequences also clearly supported our study specimens as a distinct new species.

## Conclusions

*Paramacrobiotus bengalensis* sp. nov. is a new species to science. The species is described using an integrative approach of morphometry, microscopic imaging (PCM and SEM), and genetic comparison with two DNA markers (18S rRNA and COI). The diversity of cryptic and pseudocryptic species within this group complicates taxonomic identification (Guidetti *et al.* 2019). The smaller number of morphological characteristics and variations among this group presents difficulties in identifying and defining a species from this genus exclusively based on traditional taxonomy. Recent studies that utilized integrative methods, namely, DNA sequencing combined with detailed morphological and morphometric data, have proven to be extremely useful in uncovering the evolutionary relationships within this genus and unmasking new species. However, only a few of the 45 currently recognized taxa of *Paramacrobiotus* have DNA sequences available in GenBank, which generates bias during a phylogenetic analysis and hinders species identification based on DNA. More intensive sampling of taxa of this genus with detailed morphometric data and DNA sequence data is still needed to reveal the hidden species richness within this cosmopolitan group of tardigrades.

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## Compliance with ethical standards

On behalf of all the authors, the corresponding author declares no conflict of interest, of any financial or academic nature, in this work.

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## Supplementary material

**Supp. file 1.** Raw measurements data. <https://doi.org/10.5852/ejt.2023.890.2249.9633>

**Supp. file 2.** Estimates of evolutionary divergence between sequences.

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**Supp. file 3.** Best Partitioning Scheme. <https://doi.org/10.5852/ejt.2023.890.2249.9637>

**Supp. file 4.** GenBank accession numbers for sequences of *Paramacrobotus* Guidetti, Schill, Bertolani, Dandekar & Wolf, 2009 and *Tenuibiotus* Pilato & Lisi, 2011 (outgroup) species used in this study for species delimitation analyses. <https://doi.org/10.5852/ejt.2023.890.2249.9639>

**Supp. file 5.** mPTP results. <https://doi.org/10.5852/ejt.2023.890.2249.9641>

**Supp. file 6.** Best Partitioning scheme for the COI sequences used for species delimitation analysis. <https://doi.org/10.5852/ejt.2023.890.2249.9643>

**Supp. file 7.** ASAP analysis result. <https://doi.org/10.5852/ejt.2023.890.2249.9645>

**Supp. file 8.** Estimates of evolutionary divergence between sequences. <https://doi.org/10.5852/ejt.2023.890.2249.9647>