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An integrative description of *Macrobiotus hannae* sp. nov. (Tardigrada: Eutardigrada: Macrobiotidae: *hufelandi* group) from Poland

Bernadeta NOWAK, Daniel STEC*
Institute of Zoology and Biomedical Research, Jagiellonian University, Kraków, Poland

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Abstract: A new species of the *Macrobiotus hufelandi* group is described from Poland. An integrative taxonomic approach was applied by combining morphological and morphometric analysis imaging under phase contrast and scanning electron microscopy with molecular analysis (four molecular markers) to unambiguously support the establishment of the new species. The specimens of *Macrobiotus hannae* **sp. nov.** are similar to three taxa of the *Macrobiotus hufelandi* complex: *Macrobiotus hufelandi* C.A.S. Schultze, 1834; *M. punctillus* Pilato, Binda & Azzaro, 1990; and *M. joannae* Pilato & Binda, 1983. Nevertheless, the new species differs from the first two species by the absence of cuticular bars under claws on legs I–III, better developed oral cavity armature (large, with all three bands of teeth always well visible), the presence of a subterminal constriction in the second macroplacoid, some fine peculiarities in egg ornamentation morphology, and differences in a number of morphometric characters. The new species is most similar to *M. joannae* but it can be distinguished from it by the presence of a much finer granulation on the dorsal and lateral cuticle of the body that, in contrast to *M. joannae*, is not visible in light microscopy. Our study suggests that European records of *M. joannae* are most likely invalid and they represent *M. hannae* or an unknown similar species.

Key words: 18S rRNA, 28S rRNA, COI, ITS-2, integrative taxonomy, *Macrobiotus hufelandi* group, *Macrobiotus joannae*

1. Introduction

The phylum Tardigrada comprises over 1200 species and yet remains poorly known (http://www.evozoo.unimore.it/site/home/documento1080026927.html). Over a dozen tardigrade species new for science are described each year, thereby expanding our knowledge of their biodiversity (Michalczyk and Kaczmarek, 2013). These microinvertebrates have a global distribution and inhabit a large variety of habitats from the greatest depths of the ocean to the highest mountain peaks, as well as extreme environments such as cryoconite holes (Nelson et al., 2015; Zawierucha et al., 2015). Research on the tardigrade fauna in Poland has been conducted for more than a century (Minkiewicz, 1914; Jakubski, 1915) and up to now 110 species have been reported from this country (Dastych, 1988; Gąsiorek et al., 2016; Nowak and Stec, 2017; Stec et al., 2017b; Kaczmarek et al., 2018; and literature cited therein).

The *Macrobiotus hufelandi* complex is an informal taxonomic group without taxonomic value within the genus *Macrobiotus* C.A.S. Schultze, 1834, which currently comprises 48 species (Kaczmarek and Michalczyk, 2017). The designation of a given species as belonging to the *Macrobiotus hufelandi* complex is dependent on the species meeting the following morphological criteria: porous cuticle, two macroplacoids and a microplacoid in the pharynx, and eggs, in the majority of species ornamented with inverted goblet-shaped processes that make them easily distinguishable from those of other tardigrades (Bertolani and Rebecchi, 1993; Guidetti et al., 2013; Kaczmarek and Michalczyk, 2017). To date only four species of the *Macrobiotus hufelandi* complex, for which the records are considered valid, have been reported from Poland: *Macrobiotus macrocalix* Bertolani & Rebecchi, 1993, by Kaczmarek and Michalczyk (2004); *M. polonicus* Pilato, Kaczmarek, Michalczyk & Lisi, 2003 and *M. vladimiri* Bertolani, Biserov, Rebecchi & Cesari, 2011, by Nowak and Stec (2017); and *M. sottilei* Pilato, Kiosya, Lisi & Sabella, 2012, by Kaczmarek et al. (2018). *M. hufelandi* C.A.S. Schultze, 1834 has been also

* Correspondence: daniel_stec@interia.eu

reported by many other authors (Pigoń and Węglarska, 1953; Węglarska, 1959, 1973; Pilato and Dastych, 1974; Hęciak, 1976; Węglarska and Korecka, 1983; Dastych, 1997; Michalczyk and Kaczmarek, 2003a), but following the formal redescription of *M. hufelandi* by Bertolani and Rebecchi (1993) and DNA barcoding studies on the *M. hufelandi* group (Cesari et al., 2009; Bertolani et al., 2011b; Cesari et al., 2011) all records except the neotype locality should be treated with great caution as many of them are likely to represent different species of this complex.

In this article we describe a new species of the *M. hufelandi* group, *Macrobiotus hannae* **sp. nov.**, discovered in a moss sample collected from Podlasie Province (northern Poland). We applied an integrative approach including phase contrast light microscopy (PCM) and scanning electron microscopy (SEM) observations as well as DNA sequencing (three nuclear markers: 18S rRNA, 28S rRNA, ITS-2; and one mitochondrial marker: COI) to the multifaceted delineation of the new species. Given the high similarity of the new species to *M. joannae* Pilato & Binda, 1983, described from Australia but also reported from Europe, we question the validity of the European records of the latter species.

2. Materials and methods

2.1. Sample processing and tardigrade culturing

A moss sample from soil was collected by Kasper Hlebowicz in June 2014 from a deciduous forest in Podlasie Province (northern Poland). The sample was collected and examined for terrestrial tardigrades following a protocol by Dastych (1980) modified by Stec et al. (2015). A total of 24 live tardigrades were extracted from the sample and placed in an in vitro culture. Specimens were reared on plastic petri dishes according to the protocol by Stec et al. (2015). The culture was maintained in our laboratory and examined once a week, at which time uneaten rotifers and algae were removed and replaced with fresh material. In order to perform the taxonomic analysis and specific diagnosis of this species animals and eggs were taken from the culture and split into four groups: 95 animals and 126 eggs were mounted on microscope slides in Hoyer's medium; about 20 animals and 20 egg shells were prepared for SEM imaging; four specimens were used for DNA extraction and sequencing (see below for details); and 20 gravid specimens were used for aceto-orcein staining to check the presence of the eventual hermaphroditic gonad (Bertolani, 1971; Bertolani et al., 1983; Stec et al., 2016b).

2.2. Microscopy and imaging

Specimens for light microscopy were mounted on microscope slides in a small drop of Hoyer's medium and secured with a cover slip, following the protocol by Morek et al. (2016). Slides were then dried for 5–7 days at 60 °C. Dried slides were sealed with transparent nail polish and examined under a Nikon Eclipse 50i phase contrast light microscope associated with a Nikon Digital Sight DS-L2 digital camera. In order to obtain clean and extended specimens for SEM, tardigrades were processed according to the protocol by Stec et al. (2015). In short, specimens were first subjected to a 60 °C water bath for 30 min to obtain fully extended animals, next to a water/ethanol and ethanol/acetone series, and then to CO_2 critical point drying, and finally sputter-coated with a thin layer of gold. Specimens were examined under high vacuum with a Versa 3D DualBeam Scanning Electron Microscope at the ATOMIN facility of the Jagiellonian University, Kraków, Poland. In order to establish the reproductive mode of the new species, the type population was also examined with aceto-orcein staining in accordance with Stec et al. (2016b). All figures were assembled in Corel Photo-Paint X6, ver. 16.4.1.1281. For deep structures that could not be fully focused in a single photograph, a series of 2–8 images were taken every ca. 0.2 µm and then assembled manually in Corel Photo-Paint X6, ver. 16.4.1.1281, into a single deep-focus image.

2.3. Morphometrics and morphological nomenclature

All measurements are given in micrometers. Sample size was adjusted following recommendations by Stec et al. (2016a), i.e. 30 animals and 30 eggs were measured for the accurate estimation of trait means and ranges. 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 that of Michalczyk and Kaczmarek (2003b) and Kaczmarek and Michalczyk (2017). Buccal tube length and the level of the stylet support insertion point were measured according to Pilato (1981). Buccal tube width was measured as the external and internal diameter at the level of the stylet support insertion point. Macroplacoid length sequence is given according to Kaczmarek et al. (2014). Lengths of the claw branches were measured from the base of the claw (i.e. excluding the lunula) to the top of the branch, including accessory points (Kaczmarek and Michalczyk, 2017). 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). Distance between egg processes was measured as the shortest line connecting base edges of the two closest processes (Kaczmarek and Michalczyk, 2017). Morphometric data were handled using the "Parachela" ver. 1.2 template available from the

Tardigrada Register (Michalczyk and Kaczmarek, 2013). Tardigrade taxonomy follows Bertolani et al. (2014).

2.4. Comparative material

The taxonomic key for the *M. hufelandi* group by Kaczmarek and Michalczyk (2017) was used to determine whether the isolated species had previously been described. After the species could not be identified with the key, we compared it with the original descriptions of the most similar *M. hufelandi* group species that have the *hufelandi* type oral cavity armature and *hufelandi* type egg surface, i.e. *M. hufelandi* C.A.S. Schultze, 1834; *M. punctillus* Pilato, Binda & Azzaro, 1990; and *M. joannae* Pilato & Binda, 1983. Additionally, thanks to Giovanni Pilato, who loaned slides with some paratypes and eggs of *M. joannae* to Peter Degma, who then kindly provided us with photos of the specimens and an egg, we were able to compare the morphological details between the new species and *M. joannae*.

2.5. Genotyping

The DNA was extracted from individual animals following a Chelex 100 resin (Bio-Rad) extraction method by Casquet et al. (2012) with modifications as described in detail by Stec et al. (2015). We sequenced four DNA fragments differing in mutation rates (from the most to least conservative): the small ribosome subunit (18S rRNA, nDNA), the large ribosome subunit (28S rRNA, nDNA), the internal transcribed spacer (ITS-2, nDNA), and the cytochrome oxidase subunit I (COI, mtDNA). All fragments were amplified and sequenced according to the protocols described by Stec et al. (2015). Primers and original references for specific PCR programs are listed in Table 1. Sequencing products were read with the ABI 3130xl sequencer at the Molecular Ecology Lab, Institute of Environmental Sciences of the Jagiellonian University, Kraków, Poland. Sequences were processed in BioEdit ver. 7.2.5 (Hall, 1999) and submitted to GenBank.

2.6. Comparative molecular analysis

For molecular comparisons, all published sequences of the four above mentioned markers for species of the *M. hufelandi* group were downloaded from GenBank (listed in Table 2). The sequences were aligned using the default settings (in the case of COI) and the Q-INS-I method (in the case of the nuclear markers, 18S rRNA, 28S rRNA, and ITS-2) of MAFFT version 7 (Katoh et al., 2002; Katoh and Toh, 2008) and manually checked against nonconservative alignments in BioEdit. Then the aligned sequences were trimmed to 772 (18S rRNA), 711 (28S rRNA), 300 (ITS-2), and 622 (COI) bp. All COI sequences were translated into protein sequences in MEGA7 (Kumar et al., 2016) to check against pseudogenes. Uncorrected pairwise distances were calculated using MEGA version 7.0 (Kumar et al., 2016). Although genetic distances in barcoding studies are frequently calculated in accordance with the Kimura 2 parameter (K2P) model, as proposed by Hebert et al. (2003), the more recent work by Srivathsan and Meier (2012) showed that this model of nucleotide evolution is poorly justified. Moreover, Srivathsan and Meier (2012) showed that uncorrected p-distances may provide a comparable or even a higher success rate of taxon delimitation than distances computed under the K2P. Therefore, we used basic p-distances in all of our analyses.

2.7. Data deposition

Raw morphometric measurements underlying the description of *Macrobiotus hannae* **sp. nov.** are deposited in the Tardigrada Register (Michalczyk and Kaczmarek, 2013) under www.tardigrada.net/register/00052.htm. The DNA sequences for the type population are deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank).

Table 1. Primers and references for PCR protocols for amplification of the four DNA fragments sequenced in this study.

DNA fragment	Primer name	Primer direction	Primer sequence (5'-3')	Primer source	PCR program
18S rRNA	18S_Tar_Ff1	Forward	AGGCGAAACCGCGAATGGCTC	Stec et al. (2017)	Zeller (2010)
	18S_Tar_Rr1	Reverse	GCCGCAGGCTCCACTCCTGG		
28S rRNA	28SF0001	Forward	ACCCVCYNAATTTAAGCATAT	Mironov et al. (2012)	Mironov et al. (2012)
	28SR0990	Reverse	CCTTGGTCCGTGTTTCAAGAC		
ITS-2	ITS2_Eutar_Ff	Forward	CGTAACGTGAATTGCAGGAC	Stec et al. (2018)	Stec et al. (2018)
	ITS2_Eutar_Rr	Reverse	TCCTCCGCTTATTGATATGC		
COI	LCO1490	Forward	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)	Michalczyk et al. (2012)
	HCO2198	Reverse	TAAACTTCAGGGTGACCAAAAAATCA		

Table 2. Sequences used for molecular comparisons and phylogenetic analyses of *Macrobiotus hannae* **sp. nov.** with all other species of the *Macrobiotus hufelandi* group for which DNA sequences are currently available.

DNA marker	Species	Accession number	Source
18S	*M. hufelandi*, C.A.S. Schultze, 1834	GQ849024	Giribet et al. (1996)
	M. hufelandi gr	HQ604971, FJ435738–40	Bertolani et al. (2014); Guil and Giribet (2012)
	"*M. joannae* Pilato & Binda, 1983"	HQ604974–5	Bertolani et al. (2014)
	M. kristenseni Guidetti et al., 2013	KC193577	Guidetti et al. (2013)
	M. macrocalix Bertolani and Rebecchi, 1993	HQ604976	Bertolani et al. (2014)
	M. paulinae Stec et al., 2015	KT935502	Stec et al. (2015)
	M. polypiformis Roszkowska et al., 2017	KX810008	Roszkowska et al. (2017)
	M. polonicus Pilato et al., 2003	HM187580	Wełnicz et al. (2011)
	M. sapiens Binda & Pilato, 1984	DQ839601	Bertolani et al. (2014)
	M. scoticus Stec et al., 2017	KY797265	Stec et al. (2017a)
28S	*M. hufelandi* gr	FJ435751, FJ435754–5	Guil and Giribet (2012)
	M. paulinae Stec et al., 2015	KT935501	Stec et al. (2015)
	M. polypiformis Roszkowska et al., 2017	KX810009	Roszkowska et al. (2017)
	M. scoticus Stec et al., 2017	KY797266	Stec et al. (2017a)
ITS-2	*M. paulinae* Stec et al., 2015	KT935500	Stec et al. (2015)
	M. polonicus Pilato et al., 2003	HM150647	Wełnicz et al. (2011)
	M. polypiformis Roszkowska et al., 2017	KX810010	Roszkowska et al. (2017)
	M. sapiens Binda & Pilato, 1984	GQ403680	Schill et al. (2010)
	M. scoticus Stec et al., 2017	KY797268	Stec et al. (2017a)
COI	*M.*cf. *hufelandi*	HQ876589–94, HQ876596	Bertolani et al. (2011b)
	M. hufelandi, C.A.S. Schultze, 1834	HQ876584, HQ876586–8	Bertolani et al. (2011b)
	M. kristenseni Guidetti et al., 2013	KC193575–6	Guidetti et al. (2013)
	M. macrocalix Bertolani & Rebecchi, 1993	FJ176203–17, HQ876571	Cesari et al. (2009); Bertolani et al. (2011b)
	M. paulinae Stec et al., 2015	KT951668	Stec et al. (2015)
	M. polypiformis Roszkowska et al., 2017	KX810011–2	Roszkowska et al. (2017)
	M. sandrae Bertolani & Rebecchi, 1993	HQ876566-70, HQ876572–83	Bertolani et al. (2011b)
	M. scoticus Stec et al., 2017	KY797267	Stec et al. (2017a)
	M. terminalis Bertolani & Rebecchi, 1993	JN673960, AY598775	Cesari et al. (2011); Guidetti et al. (2005)
	M. vladimiri Bertolani et al., 2011	HM136931–4, HQ876568	Bertolani et al. (2011a, 2011b)

3. Results

3.1. Taxonomic account of the new species

Phylum: Tardigrada Doyère, 1840

Class: Eutardigrada Richters, 1926

Order: Parachela Schuster, Nelson, Grigarick & Christenberry, 1980

Superfamily: Macrobiotoidea Thulin, 1928 (in Marley et al., 2011)

Family: Macrobiotidae Thulin, 1928

Genus: *Macrobiotus* C.A.S. Schultze, 1834

***Macrobiotus hannae* sp. nov.** (Tables 3 and 4; Figures 1A–1E, 2A–2F, 3A–3E, 4A–4B, 5A–5D, 6A–6D, 7A–7F, 8A–8B)

3.2. Material examined

A total of 139 animals (including 4 in simplex stage) and 146 eggs. Specimens mounted on microscope slides in Hoyer's medium (95 animals + 126 eggs), fixed on SEM stubs (20 + 20), and processed for DNA sequencing (4 + 0) and aceto-orcein staining (20 + 0).

Table 3. Measurements [in µm] of selected morphological structures of individuals of *Macrobiotus hannae* **sp. 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		Holotype	
		µm	*pt*	µm	*pt*	µm	*pt*	µm	*pt*
Body length	30	341–760	889–1288	640	1133	93	85	638	1111
Buccal tube									
Buccal tube length	30	38.4–62.1	–	56.2	–	5.4	–	57.4	–
Stylet support insertion point	30	31.1–50.3	80.3–83.8	45.9	81.6	4.4	0.9	46.1	80.3
Buccal tube external width	30	6.0–10.7	14.9–19.4	9.6	17.0	1.0	0.9	10.1	17.6
Buccal tube internal width	30	4.4–8.3	11.1–16.0	7.2	12.7	0.8	0.9	7.2	12.6
Ventral lamina length	23	24.2–39.8	56.3–65.8	34.7	61.7	3.6	2.2	35.2	61.2
Placoid lengths									
Macroplacoid 1	30	12.2–22.2	30.5–38.9	19.4	34.5	2.5	1.9	19.9	34.7
Macroplacoid 2	30	6.8–14.9	17.7–24.8	12.2	21.5	1.8	1.5	12.5	21.8
Microplacoid	30	3.5–9.3	9.1–15.4	7.0	12.4	1.1	1.2	7.6	13.2
Macroplacoid row	30	20.2–37.7	50.8–62.4	33.1	58.7	4.3	3.1	34.1	59.4
Placoid row	30	25.2–46.6	65.6–78.9	41.1	72.9	5.3	3.4	42.0	73.1
Claw 1 lengths									
External primary branch	27	8.9–16.5	23.0–27.8	14.3	25.3	1.6	1.3	14.5	25.2
External secondary branch	27	7.4–13.6	16.4–23.3	11.4	20.0	1.5	1.5	12.0	20.9
Internal primary branch	25	10.5–14.9	20.6–26.1	13.3	23.2	1.2	1.2	12.7	22.1
Internal secondary branch	23	8.0–12.5	15.5–21.4	10.7	18.6	1.2	1.5	10.2	17.8
Claw 2 lengths									
External primary branch	27	11.0–17.5	24.1–29.8	15.1	26.7	1.4	1.4	15.6	27.2
External secondary branch	21	9.3–14.0	18.6–23.9	11.8	20.9	1.2	1.3	11.5	20.0
Internal primary branch	25	10.0–14.7	21.6–30.3	13.3	23.6	1.1	1.7	12.7	22.1
Internal secondary branch	22	8.5–12.3	16.6–20.8	10.6	18.6	1.1	1.3	9.6	16.7
Claw 3 lengths									
External primary branch	27	10.1–17.1	24.6–28.9	15.0	26.8	1.8	1.1	15.3	26.6
External secondary branch	25	9.0–13.4	18.9–23.4	11.8	20.9	1.2	1.0	12.3	21.3
Internal primary branch	26	8.4–5.2	21.8–26.3	13.2	23.5	1.7	1.3	13.5	23.5
Internal secondary branch	24	8.4–12.6	16.9–20.9	10.9	19.1	1.3	1.2	11.4	19.9
Claw 4 lengths									
Anterior primary branch	25	9.9–18.8	25.9–32.1	16.2	29.0	2.0	1.7	16.4	28.5
Anterior secondary branch	22	6.7–14.6	17.4–24.5	12.1	21.8	1.8	1.8	12.3	21.3
Posterior primary branch	26	10.5–19.8	26.1–33.4	16.6	29.7	2.0	1.9	16.9	29.4
Posterior secondary branch	12	7.4–15.0	19.2–25.5	12.2	22.5	2.2	1.9	?	?

Table 4. Measurements in µm of selected morphological structures of the eggs of *Macrobiotus hannae* **sp. nov.** mounted in Hoyer's medium (N – number of eggs/structures measured, Range – the smallest and the largest structure among all measured specimens; SD – standard deviation).

Character	N	Range	Mean	SD
Egg bare diameter	30	88.6–109.2	96.6	4.6
Egg full diameter	30	103.5–124.6	110.8	5.3
Process height	90	5.4–9.7	7.7	1.1
Process base width	90	4.3–7.8	6.0	0.8
Process base/height ratio	90	63%–95%	78%	7%
Terminal disc width	90	4.4–6.7	5.3	0.4
Distance between processes	90	4.0–8.1	5.9	0.7
Number of processes on the egg circumference	30	25–30	27.4	1.4

3.3. Description of the new species

3.3.1. Animals (measurements and statistics in Table 3)

Body transparent in juveniles and white in adults, after fixation in Hoyer's medium always transparent (Figure 1A). Eyes present. Round and oval pores (0.30–0.55 µm in diameter), scattered randomly on the entire cuticle (on the ventral side of the body distributed more sparsely) (Figures 1B–1E), including the external and internal surface of all legs. Extremely small cuticular granulation on the entire body present but visible only under SEM (Figures 1C–1E). The size of these microgranules, with diameters ranging from 0.05 to 0.07 µm, is below light microscope resolution. Evident granulation on external surface of all legs visible under PCM and SEM (0.15–0.45 µm in diameter) (Figures 2A–2D). Under PCM the granulation is seen as dark dots (Figures 2A and 2B) and under SEM as an aggregation of microgranules (Figures 2C and 2D). A cuticular pulvinus-like bulge/fold is present on the internal surface of all legs I–III (Figures 2E and 2F, filled arrowheads). This structure is visible only if the legs are fully extended and well oriented on the slide or SEM stubs.

Mouth anteroventral. Buccopharyngeal apparatus of the *Macrobiotus* type, with the ventral lamina and ten small peribuccal lamellae followed by six buccal sensory lobes (Figures 3A, 4A, and 4B). An irregular ring of pores, visible in SEM and only rarely in PCM, is present around the mouth opening, immediately behind the peribuccal sensory lobes. Under PCM the oral cavity armature is of the *hufelandi* type – three bands of teeth are always visible (Figures 3B and 3C). The first band of teeth is composed of numerous extremely small cones arranged in four to six rows situated anteriorly in the oral cavity, just behind the bases of the peribuccal lamellae (Figures 3B, 3C 4A, and 4B, filled indented arrowhead). The second band of teeth is situated between the ring fold and the third band of teeth and comprises 4–5 rows of small cones, slightly bigger than those of the first band (Figures 3B, 3C 4A, and 4B, empty indented arrowhead). 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 (Figures 3B, 3C, 4A, and 4B). The third band of teeth is discontinuous and divided into the dorsal and the ventral portion. Under PCM, the dorsal teeth are seen as three distinct transversal ridges whereas the ventral teeth appear as two separate lateral transversal ridges, between which a roundish median tooth is visible (Figures 3B and 3C). In SEM both dorsal and ventral teeth are also clearly distinct (Figures 4A and 4B, lateral teeth labeled "L", median teeth labeled "M"). Under SEM the margins of the dorsal teeth are slightly serrated (Figure 4A), whereas margins of ventral teeth are smooth (Figure 4B). Pharyngeal bulb spherical, with triangular apophyses, two rod-shaped macroplacoids, and a large triangular microplacoid (Figure 3A). The macroplacoid length sequence is 2 < 1. The first and the second macroplacoid have a distinct constriction, centrally and subterminally, respectively (Figures 3D and 3E).

Claws small and slender, of the *hufelandi* type (Figures 5A–5D). Primary branches with distinct accessory points, a long common tract, and an evident stalk connecting the claw to the lunula (Figures 5A–5D). Lunulae on legs I–III smooth (Figures 5A and 5C), whereas a faint dentation is present on lunules on legs IV (Figures 5B and 5D). A cuticular bar is present under claws I–III (Figure 2E, empty arrow head, and Figure 5A, filled arrowhead), whereas a horseshoe-shaped structure connects the anterior and posterior lunules on leg IV (Figure 5B, empty arrowhead).

3.3.2. Eggs (measurements and statistics in Table 4)

Laid freely, white and spherical (Figures 6A, 6B, and 7A). The surface between processes of the *hufelandi* type, i.e. chorion surface, covered by evident reticulum (Figures 6D and 7B–7F). The reticulation is uniform over the entire surface. There are several rows of pores between processes and the mesh walls are often wider than the pore diameter

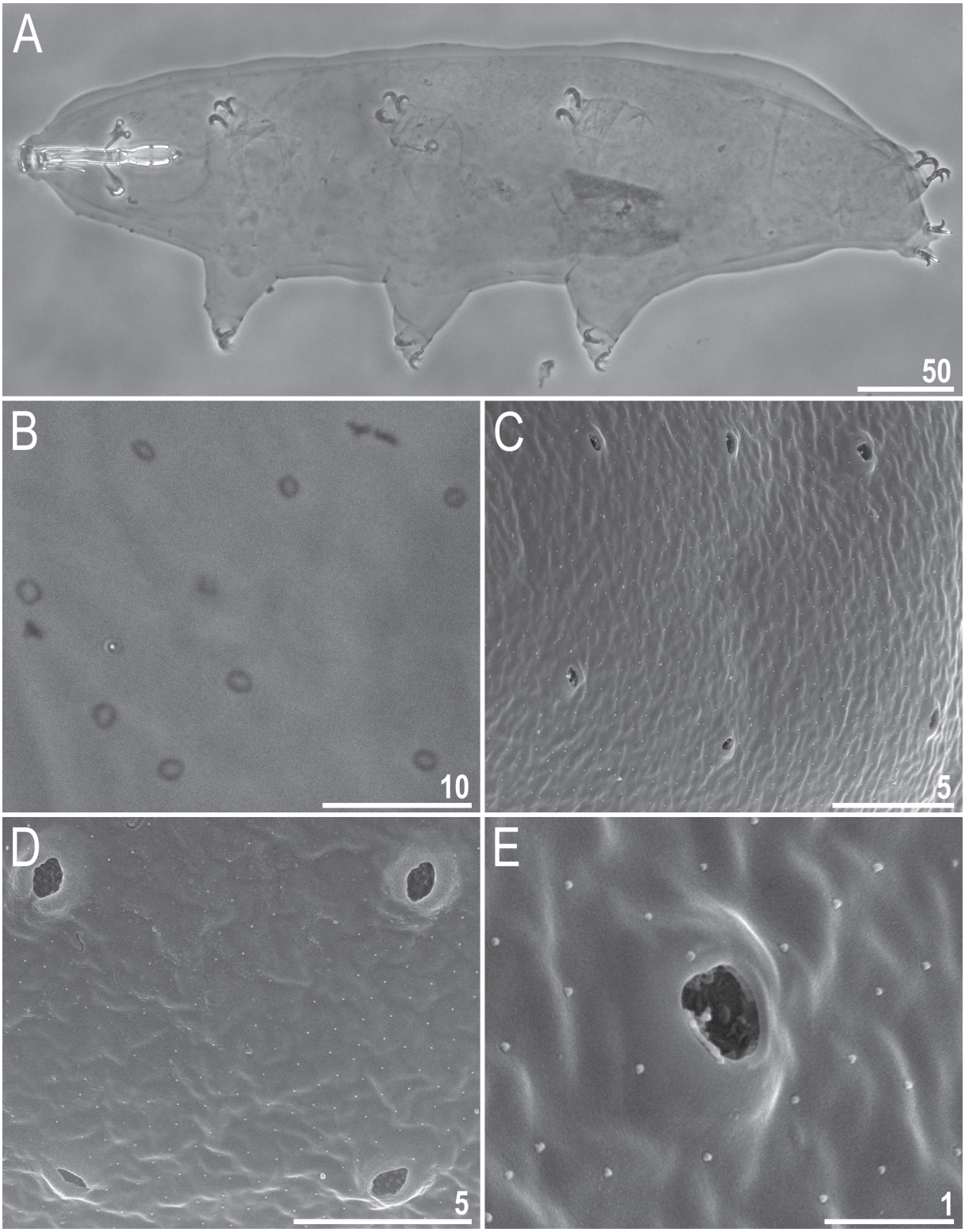

Figure 1. *Macrobiotus hannae* **sp. nov.** – habitus and cuticle morphology: A – dorsoventral projection (holotype, Hoyer's medium, PCM); B – pores in the cuticle on the dorsal side of the body seen in PCM (paratype); C–E – cuticular pores and fine granulation seen in SEM on the dorsal side of the body (paratype). Scale bars in μm.

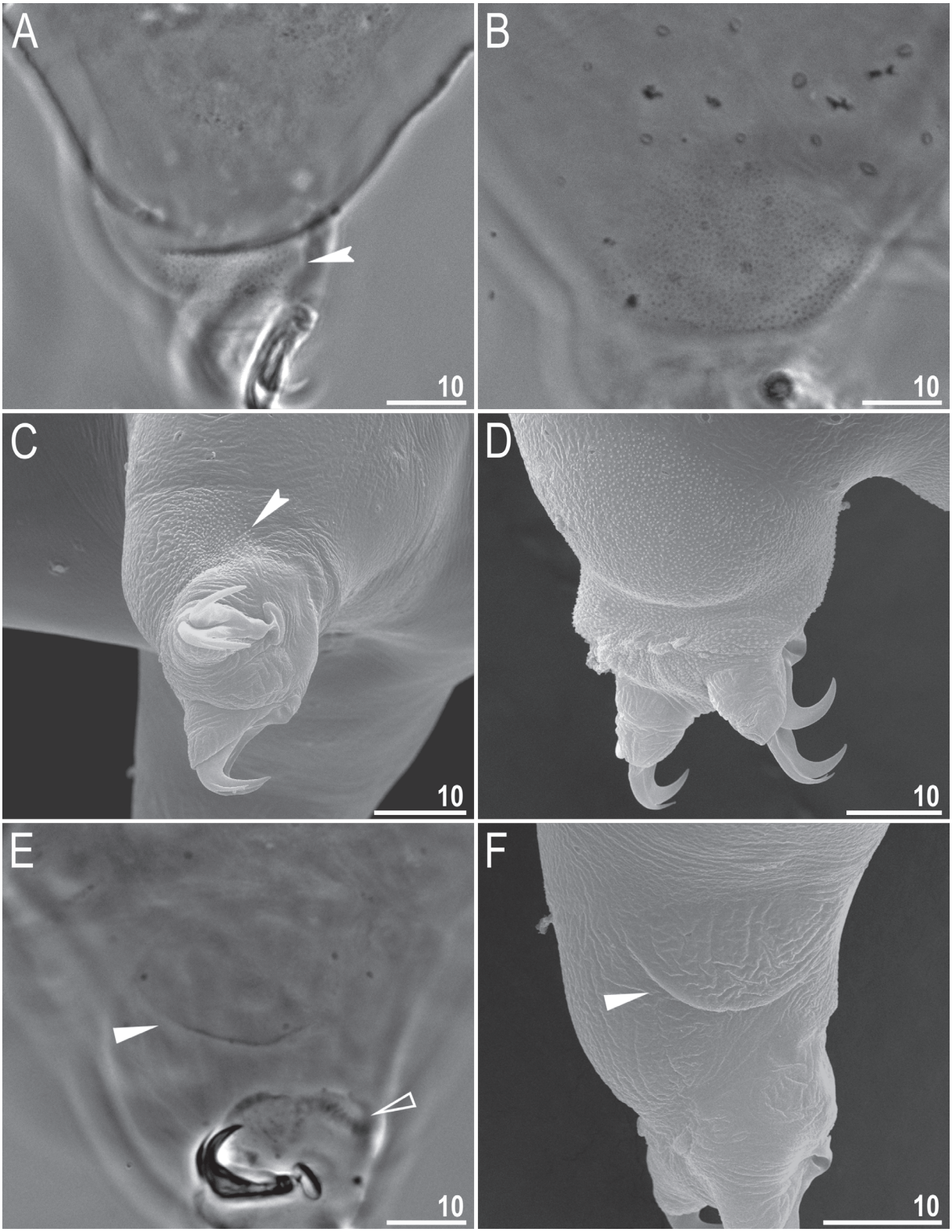

Figure 2. *Macrobiotus hannae* **sp. nov.** – cuticular structures on legs: A – granulation on leg III (paratype, PCM); B – granulation on leg IV (holotype, PCM); C – granulation on leg I (paratype, SEM); D – granulation on leg IV (paratype, SEM); E – cuticular bulge resembling pulvinus-like structure on the internal surface of leg III (paratype, PCM); F – cuticular bulge resembling pulvinus-like structure on the internal surface of leg I (paratype, SEM); filled indented arrowheads indicate the granulation on the external surface of leg III and I, respectively; filled arrowheads indicate the cuticular bulge whereas empty arrowhead indicates faint cuticular bar under the claws. Scale bars in μm.

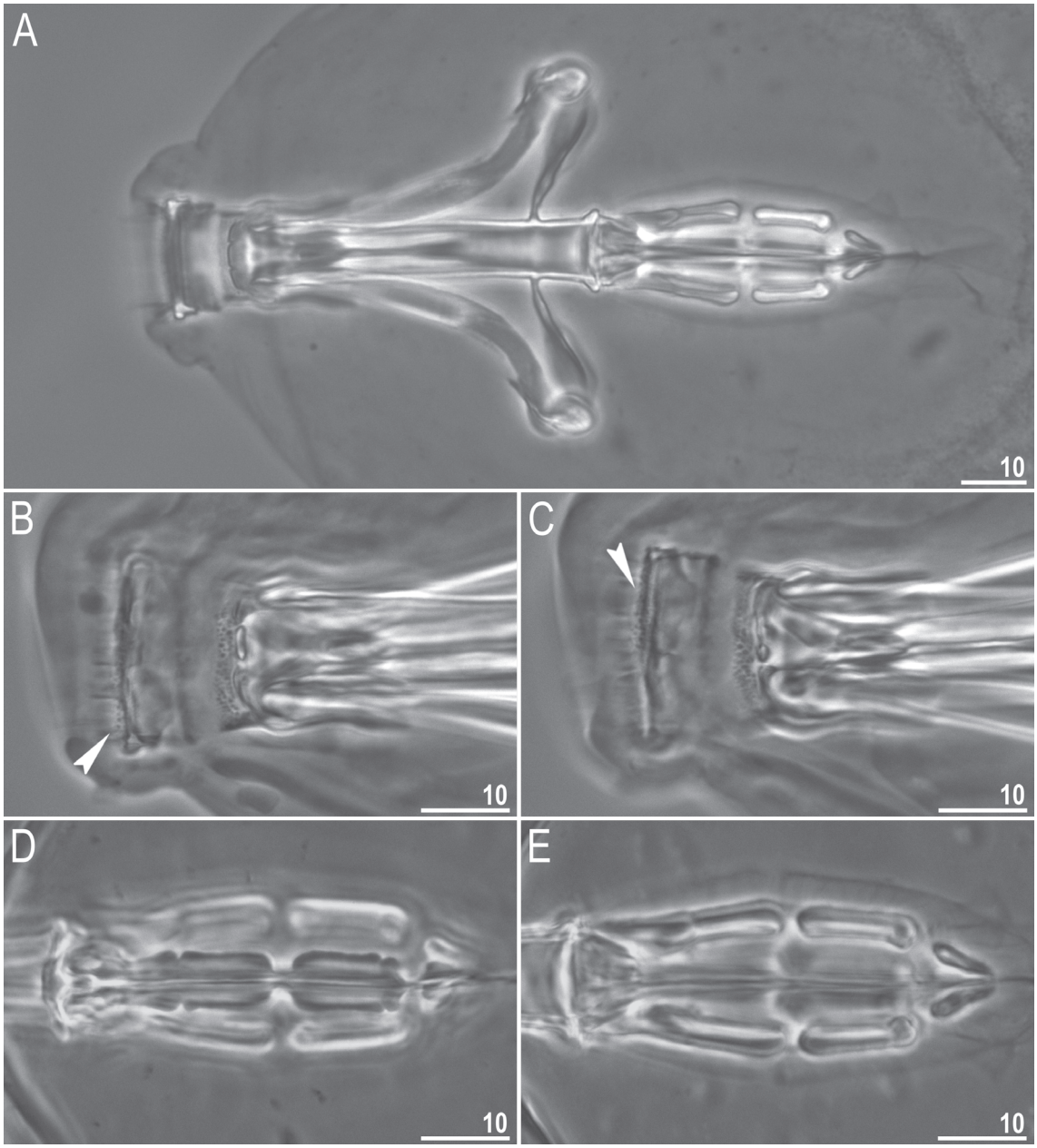

Figure 3. *Macrobiotus hannae* **sp. nov.** – buccal apparatus and the oral cavity armature seen in PCM (all paratypes): A – dorsoventral projection of the entire buccal apparatus; B–C – oral cavity armature of the *hufelandi* type (all three bands of teeth visible), dorsal and ventral view, respectively; D–E – placoid morphology, ventral and dorsal view, respectively. Filled indented arrowheads indicate teeth of the first band. Scale bars in μm.

(Figures 6D and 7B–7D). The pores in the reticulum are circular or slightly oval (0.3–0.7 μm in diameter) and under SEM almost all pores are seen to contain one or more small round or elongate granules (Figures 7C–7E). Processes are in the shape of inverted goblets with slightly concave conical trunks and well-defined terminal discs (Figures 6A–6D and 7A, 7B, 7D–7F). On the process trunk faint annulations are visible under SEM (Figures 7B, 7D, and 7E). Terminal discs are cog-shaped, with a concave central area and 10–18 distinct teeth (Figures 6D and 7B, 7D–7F). Terminal discs, and especially their teeth, are covered by aggregations of small granules (visible only under SEM), which probably serve to enhance the adhesive properties of the egg processes (Figures 7E and 7F).

3.3.3. Reproduction

The type population of *M. hannae* **sp. nov.** is hermaphroditic. In each of the analyzed adult gravid individuals, two types of gametes have been observed.

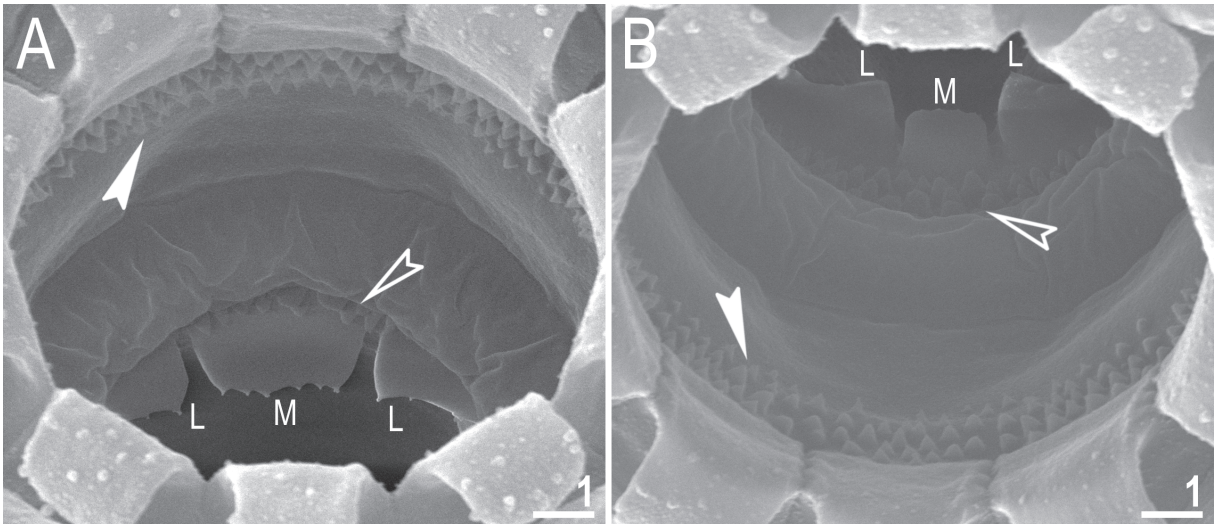

Figure 4. *Macrobiotus hannae* **sp. nov.** – the oral cavity armature of a single paratype seen in SEM from different angles, A – dorsal side; B – ventral side. Filled indented arrowheads indicate teeth of the first band, empty indented arrowheads indicate teeth of the second band, the ridges of the third band are marked with "M" (median tooth) and "L" (lateral teeth). Scale bars in μm.

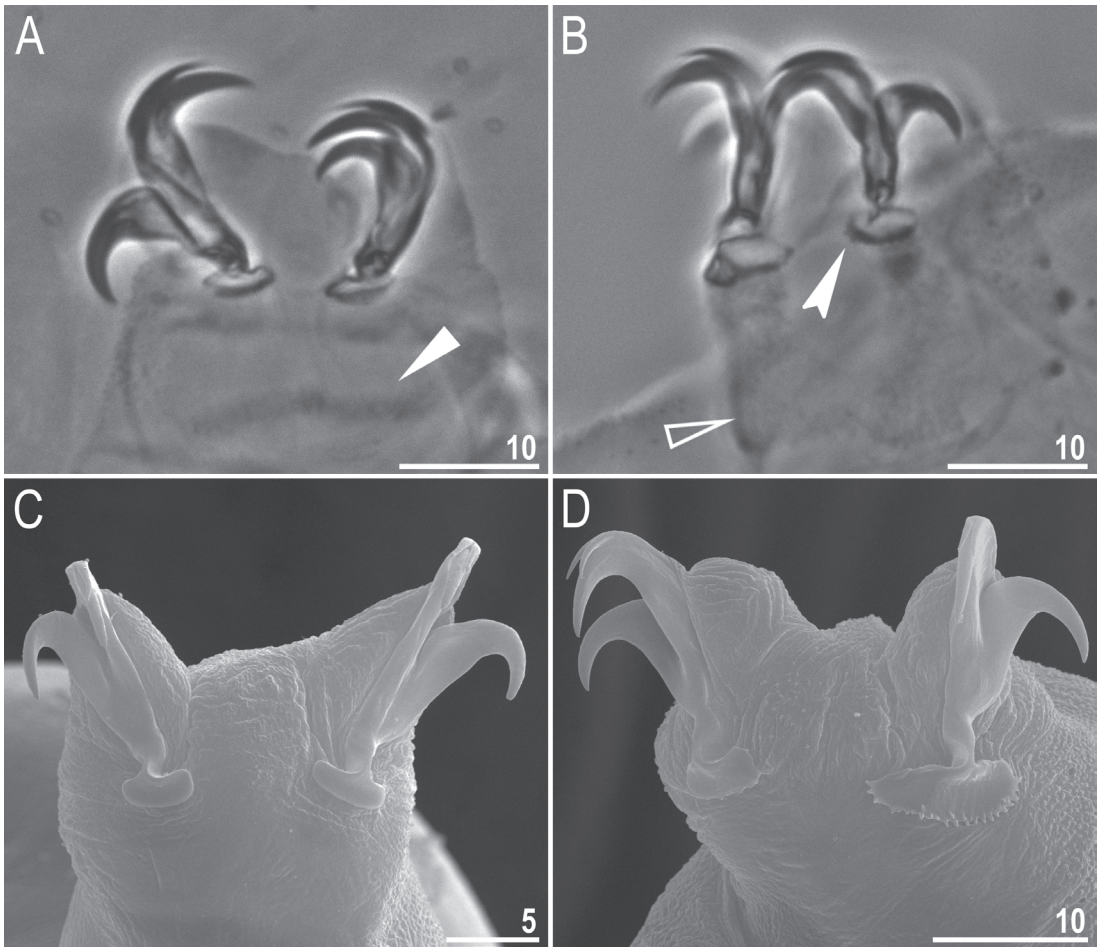

Figure 5. *Macrobiotus hannae* **sp. nov.** – claws: A–B – claws II (holotype) and IV (paratype) seen in PCM, with smooth and slightly dentate lunules respectively; C–D – claws I and IV seen in SEM, with smooth and slightly dentate lunules respectively. Filled flat arrowhead indicates a cuticular bar, filled indented arrowhead indicates indentation in lunules IV, empty arrowhead indicates the horseshoe structure connecting the anterior and the posterior claw. Scale bars in μm.

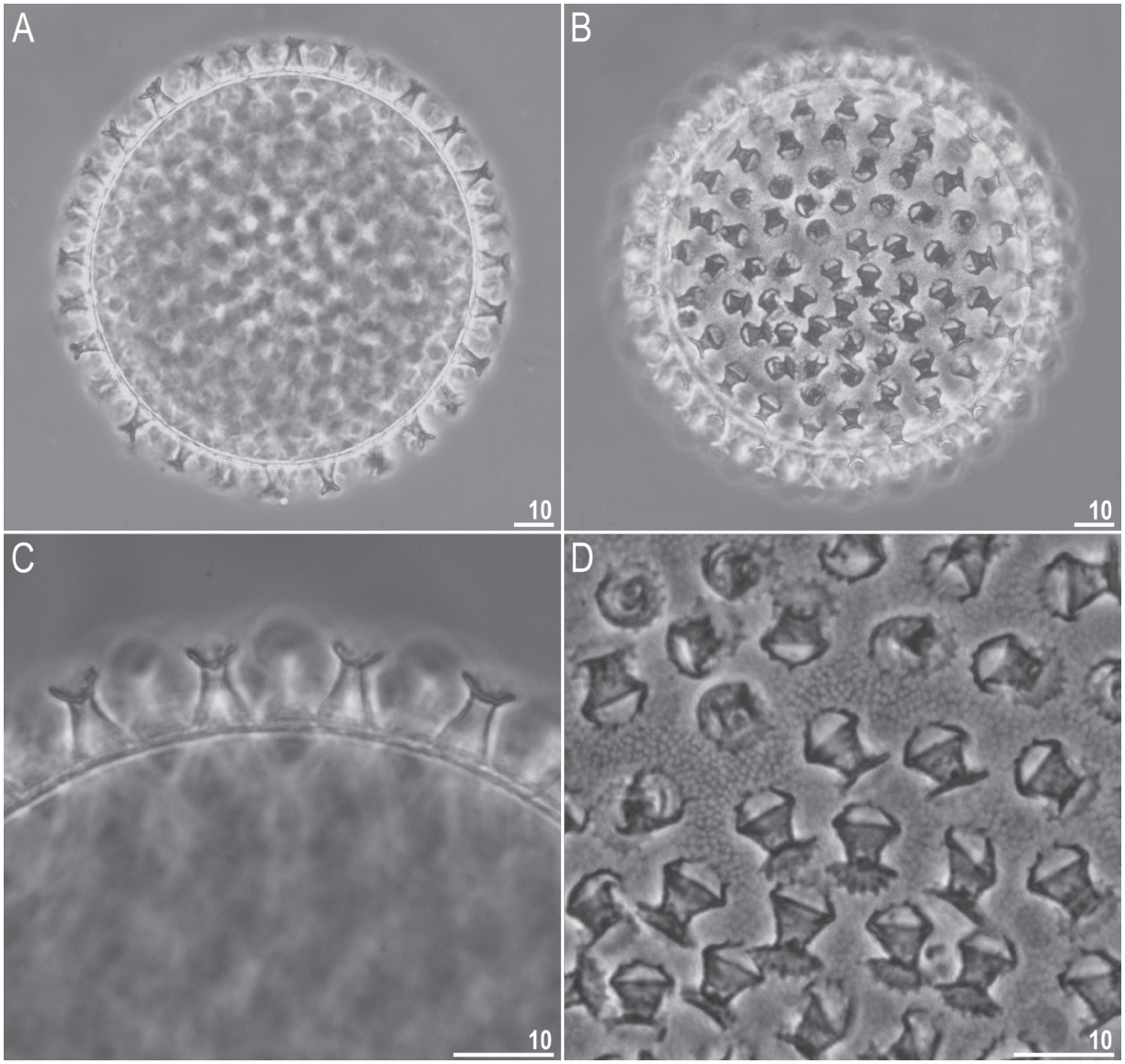

Figure 6. *Macrobiotus hannae* **sp. nov.** – egg seen in PCM: A – midsection under 400× magnification; B – surface under 400× magnification; C – midsection, to show processes, under 1000× magnification; D – surfaces under 1000× magnification. Scale bars in µm.

Aceto-orcein staining revealed the ovotestis filled with spermatozoa (Figures 8A and 8B) and developing oocytes. The hermaphroditism was independently confirmed by transmission electron microscope analysis (Izabela Poprawa, personal communication).

3.3.4. DNA sequences

We obtained very good quality sequences for all four molecular markers from all four analyzed specimens (paragenophores). DNA sequences of all markers were represented by single private haplotypes:

The **18S rRNA** sequence (GenBank: MH063922), 1035 bp long.

The **28S rRNA** sequence (GenBank: MH063924), 758 bp long.

The **ITS-2** sequence (GenBank: MH063923), 429 bp long.

The **COI** sequence (GenBank: MH057764), 657 bp long.

3.4. Type locality

Poland, Podlasie Province; moss growing on soil in deciduous forest: 53°20′39″N, 22°51′13″E; 139 m a.s.l.; coll. 05.2016 by Kasper Hlebowicz.

3.5. Etymology

We take great pleasure in dedicating this new species to the friend of the second author, Hanna Tutaj, who is a PhD student in the Institute of Environmental Sciences, Jagiellonian University, Kraków, Poland.

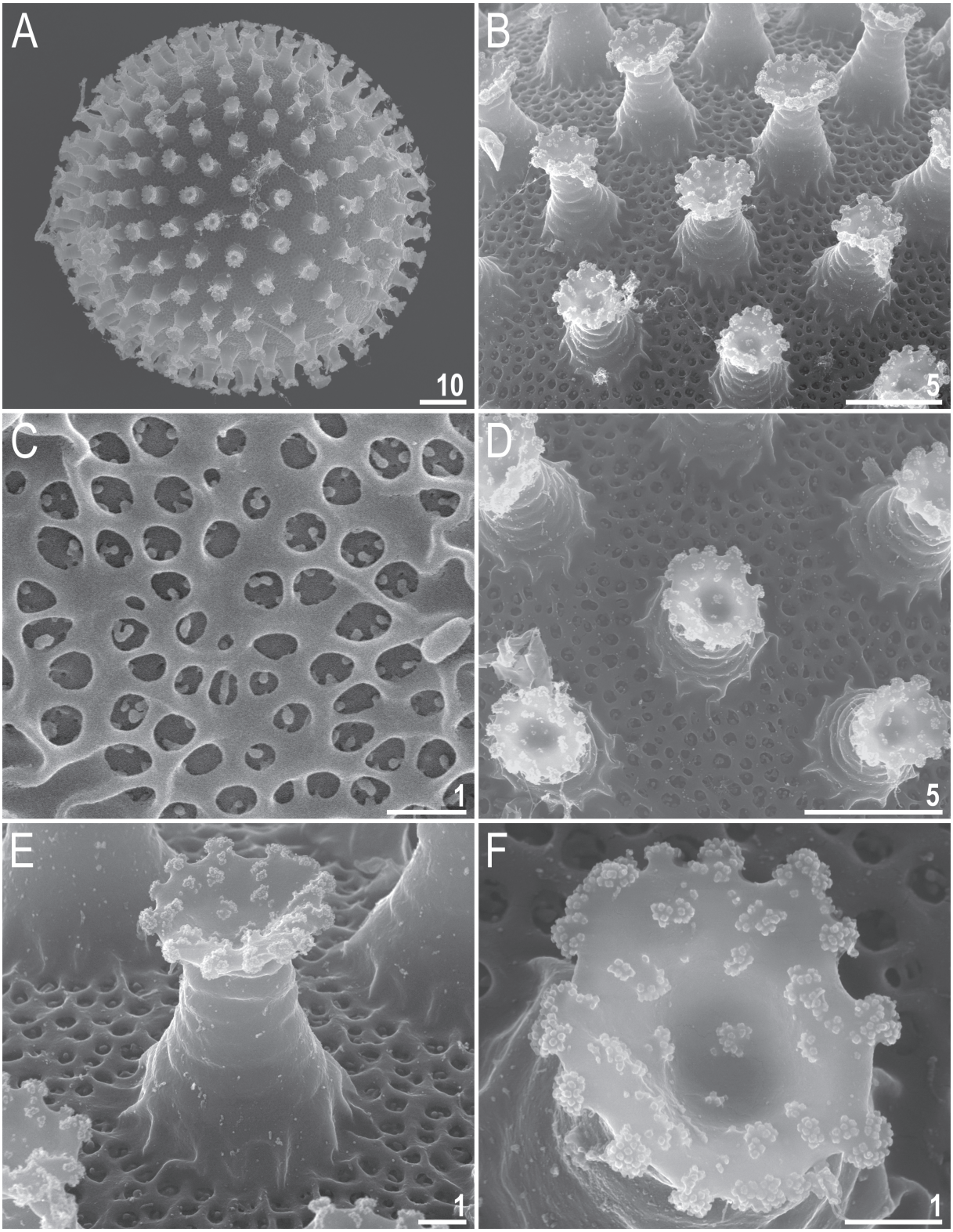

Figure 7. *Macrobiotus hannae* **sp. nov.** – egg chorion morphology seen in SEM: A – entire egg with faintly visible reticulation on the surface between processes; B–D – details of reticulation and processes arrangement on the egg surface; E–F – zoom on a single egg process and terminal disc respectively. Scale bars in μm.

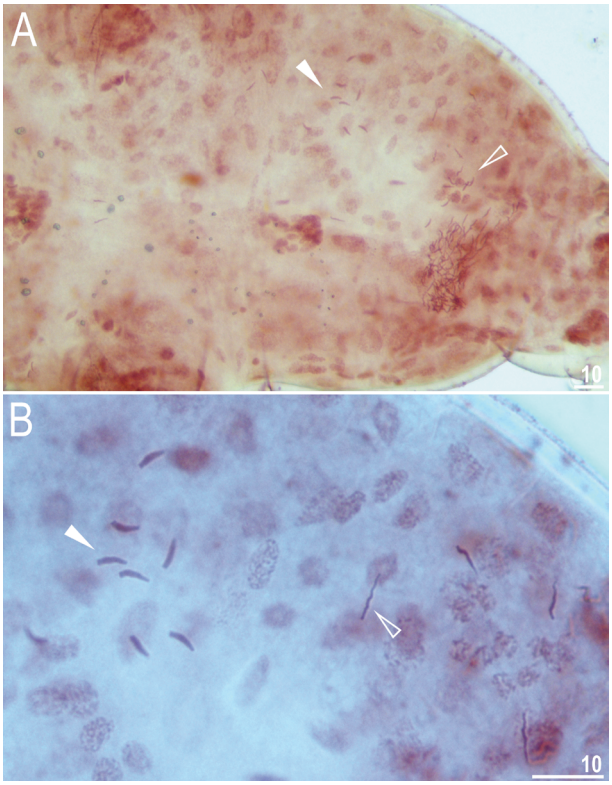

Figure 8. *Macrobiotus hannae* **sp. nov.** – ovotestis: A – general view, note the cluster of fully developed spermatozoa at the posterior end of the gonad as well as sparsely distributed spermatozoa and their earlier developmental at the central portion of the gonad; B – zoomed view of spermatozoa sparsely distributed within the ovotestis. Filled arrowheads indicate spermatozoa in early developmental stage, empty arrowheads indicate fully developed, mature spermatozoa. Scale bars in µm.

3.6. Type depositories

Holotype: slide PL.010.01, 73 paratypes (slides: PL.010/*, where the asterisk can be substituted by any of the following numbers, 2–31) and 88 eggs (slides: PL.010/*: 39–46, 51–54) are deposited at the Institute of Zoology and Biomedical Research, Jagiellonian University, Gronostajowa 9, 30-387, Kraków, Poland, and 21 paratypes (slides: PL.010/*: 32–38) and 38 eggs (slides: PL.010/*: 47–50) are deposited at the Department of Animal Taxonomy and Ecology, Institute of Environmental Biology, Adam Mickiewicz University, Umultowska 89, 61-614 Poznań, Poland.

3.7. Phenotypic differential diagnosis

By having the oral cavity armature and egg shell ornamentation of the *hufelandi* type as well as three well-defined, separate ridges in the dorsal portion of the third band of teeth in the oral cavity, the new species is similar to three species from the *Macrobiotus hufelandi* complex but differs specifically from:

- *M. joannae*, reported from its type locality in Australia (Pilato and Binda, 1983) (Figures 9A–9F, 10A, and 10B) and several uncertain localities in central, eastern, and southeastern Russia (Biserov, 1990) and from Italy (Bertolani et al., 2014), by: an extremely small and scarce granulation on the entire dorsolateral cuticle, which is visible only under SEM (large and dense granulation on the dorsolateral cuticle clearly visible under light microscope in *M. joannae*, Figures 9C and 9D), and by weakly developed teeth on the lunules under claws IV (well-developed teeth lunules IV in *M. joannae*, Figure 9F).
- *M. hufelandi*, reported from all continents, although with current knowledge only the type locality in Germany should be considered as valid (Bertolani and Rebecchi, 1993; Bertolani et al., 2011b), by: the presence of cuticular bars under claws I–III (bars absent in *M. hufelandi*); better developed oral cavity armature (several rows of large and clearly visible teeth in the first and second band of teeth in the new species vs. several rows of smaller and less obvious teeth in the first and second band of teeth in *M. hufelandi*); a different morphology of the reticulation on the egg surface (smaller mesh size, several rows of pores in the reticulum between processes, mesh bars often wider than pore diameter, pores in the reticulum almost circular in the new species vs. bigger mesh size, often only two rows of pores in the reticulum between processes, mesh bars clearly thinner than pore diameter, and pores in the reticulum more ovoid in *M. hufelandi*); a different morphology of terminal discs of egg processes (terminal discs covered by aggregations of small granules in the new species vs. terminal discs without any granulation in *M. hufelandi*). *Remarks*. Although there are no quantitative (morphometric) differences between the new species and *M. hufelandi*, and only qualitative are present, the species distinction is well supported by genetic differences. Genetic distance between the new species and neotype population of *M. hufelandi* ranges from 18.3% to 18.4%, which is much more than the arbitrarily adopted threshold for species delineation in tardigrades (Cesari et al., 2009).
- *M. punctillus*, reported only from its type locality in Chile (Pilato et al., 1990), by: the lack of body granulation visible under light microscope (cuticular granulation clearly present over the whole body under light microscope in *M. punctillus*); the presence of cuticular bars under claws I–III (cuticular bars absent in *M. punctillus*); the presence of a subterminal constriction in the second macroplacoid (the constriction absent in *M. punctillus*); slightly dentate lunules under claws IV (lunules IV smooth in *M. punctillus*); a slightly shorter second macroplacoid (6.8–8.3 µm [*pt = 17.7–18.1*] in

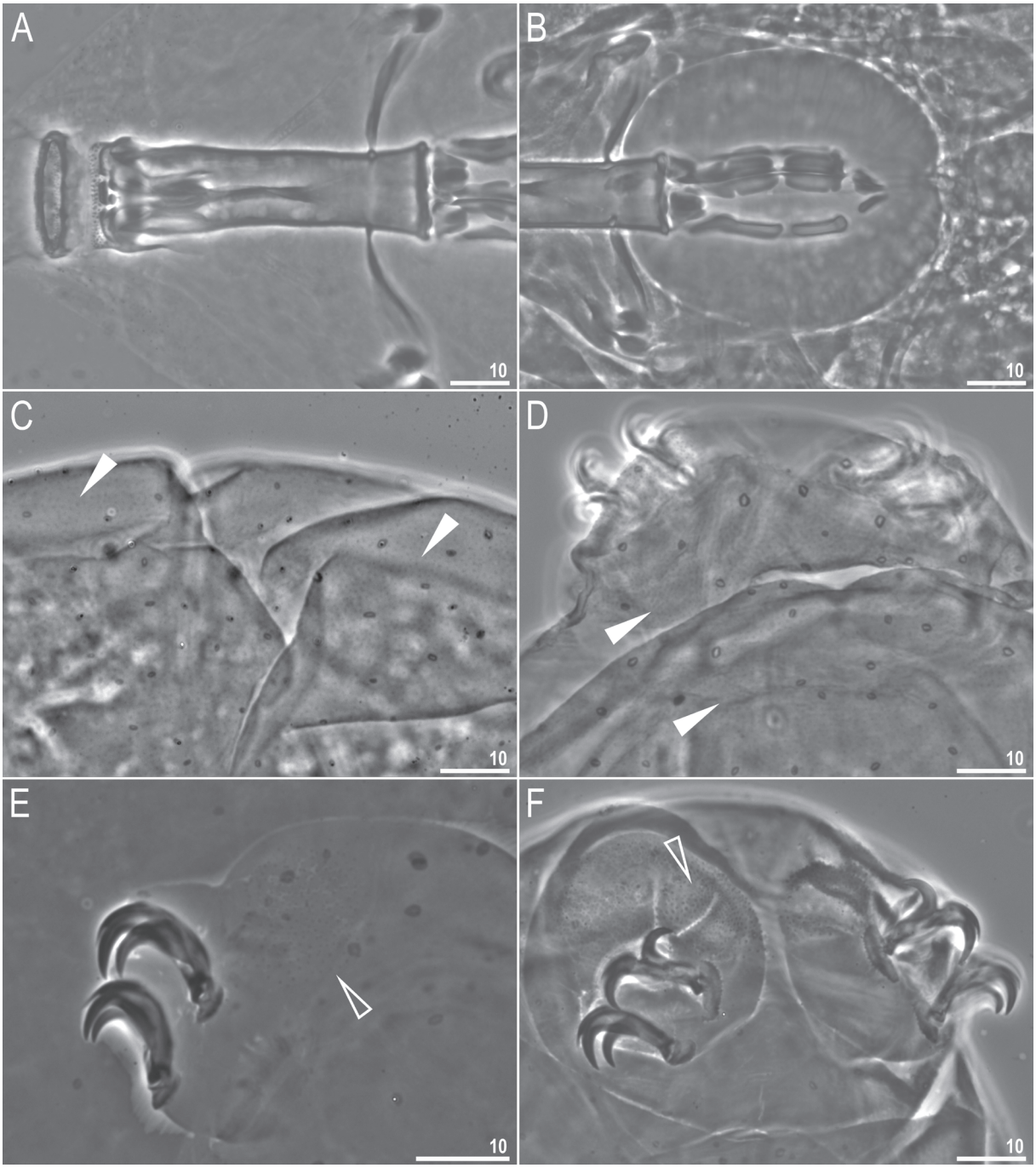

Figure 9. *Macrobiotus joannae* Pilato & Binda, 1983 – details of animal morphology (paratypes): A–B – buccal tube with oral cavity armature and pharyngeal bulb with placoids seen in PCM respectively; C–D – granulation on the dorsolateral cuticle in the middle of the body and on the dorsal cuticle in the caudal region respectively; E–F – claws and granulation on the II and IV pair of legs, respectively. Filled arrowheads indicate granulation on the dorsolateral cuticle, empty arrowheads indicate leg granulations. Scale bars in μm.

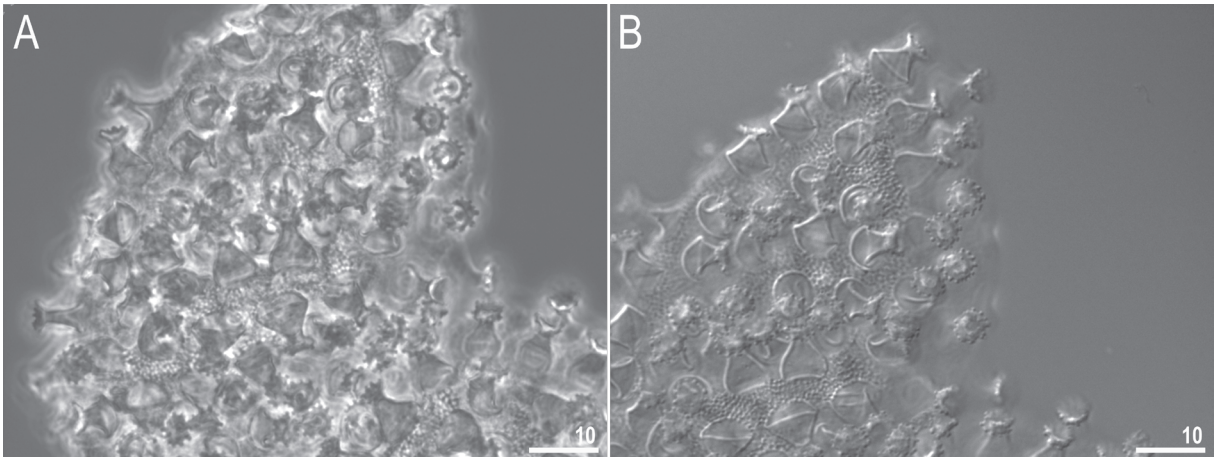

Figure 10. *Macrobiotus joannae* Pilato & Binda, 1983 – details of the egg: A – surfaces under 1000× magnification seen in PCM; B – surface under 1000× magnification seen in DIC. Scale bars in μm.

two specimens of the new species with body lengths 341 and 431 μm respectively vs. 9.6 μm [*pt = 22.8*] in one specimen of *M. punctillus* with body length 397 μm); a slightly longer microplacoid (3.5–5.2 μm [*pt = 9.1–11.5*] in two specimens of the new species with body lengths 341 and 431 μm respectively vs. 3.1 μm [*pt = 7.3*] in one specimen of *M. punctillus* with body length 397 μm); larger egg diameters (bare 88.6–109.2 μm and full 103.5–124.6 μm in the new species vs. 70.0–71.0 μm and 83.0–84.0 μm in *M. punctillus*); a lower number of processes on egg circumference (25–30 in the new species vs. 32–33 in *M. punctillus*); a larger diameter of the terminal discs on egg processes (4.4–6.7 μm in the new species vs. 3.7–3.9 μm in *M. punctillus*).

3.8. Genotypic differential diagnosis

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

- **18S rRNA**: 0.0%–4.5% (2.4% on average), with the identical sequence being "*M. joannae*" from Italy (HQ604974–5) and the least similar being *M. polypiformis* Roszkowska et al., 2017 from Ecuador (KX810008);
- **28S rRNA**: 3.9%–12.5% (7.6% on average), with the most similar being an undetermined *M. hufelandi* group species from Spain (FJ435751, FJ435754–5) and the least similar being *M. polypiformis* from Ecuador (KX810009);
- **COI**: 17.9%–26.0% (19.9% on average), with the most similar being *M. sandrae* Bertolani and Rebecchi, 1993 from Germany (HQ876577–9 and HQ876581) and the least similar being *M. polypiformis* from Ecuador (KX810012).
- **ITS-2**: 22.4%–33.3% (30.1% on average), with the most similar being *M. polonicus* from Poland (HM150647) and the least similar *M. scoticus* Stec et al., 2017 from Scotland (KY797268).

4. Discussion

Macrobiotus hannae **sp. nov.**, characterized in this paper, is very similar to *M. joannae*, a species of the *M. hufelandi* group originally described from Australia by Pilato and Binda (1983) and later reported from Russia by Biserov (1990) and from Italy by Bertolani et al. (2014). However, the description of *M. hannae* **sp. nov.** from Poland questions the European reports of *M. joannae* and suggests they may, in fact, be misidentified records of the new species. This hypothesis is supported by SEM observations of the Russian individuals and by the 18S rRNA sequence of the Italian population. Specifically, Biserov (1990) showed that the Russian specimens exhibit cuticular granulation that is so fine that it is visible only under SEM (Figure 4A therein). Thus, the granulation is similar to that in *M. hannae* **sp. nov.** (Figures 1B–1E) rather than to the large and dense granulation in *M. joannae* (Figures 9C and 9D). Moreover, Biserov (1990) described the same microgranule aggregations on the terminal disc of the egg processes (Figures 4B and 4C therein) as those we show in *M. hannae* **sp. nov.** (Figures 7E and 7F). Finally, Biserov (1990) also noted that the lunule IV indentation, similarly to that in *M. hannae* **sp. nov.**, was less well developed in the Russian populations than in *M. joannae*. Unfortunately, no phenotypic information was provided in the case of the Italian population attributed to *M. joannae* by Bertolani et al. (2014). However, the 18S rRNA sequence for the Italian "*M. joannae*" (HQ604974–5; Bertolani et al. (2014)) is identical to the type 18S rRNA sequence for *M. hannae* **sp. nov.** (MH063922). Although the 18S rRNA

is a conservative marker and identical haplotypes may in principle be shared by closely related species, the lack of even a single point mutation suggests that the Italian population represents *M. hannae* **sp. nov.** described from a locality about 1350 km away and known also from several other European localities (Biserov, 1990) rather than *M. joannae* described from a locality about 16,300 km away. Thus, in light of our findings, the geographic distribution of the new species should be considered as encompassing the type locality in Poland, the Russian localities reported by Biserov (1990), and most likely also the Italian locality reported by Bertolani et al. (2014). In other words, with the currently available data, *M. hannae* **sp. nov.** should be considered a European species. At the same time, the confirmed geographic distribution of *M. joannae* seems to be limited to the type locality in Australia. Because of the morphological similarities (Figures 9A, 9B, 9E, 10A, and 10B), as well as the same condition of sexuality (hermaphroditism) (Bertolani et al., 1983; Pilato and Binda, 1983) (Figures 8A and 8B), between *M. hannae* **sp. nov.** and *M. joannae*, it can be also hypothesized that these are sibling species. However, this hypothesis can only be tested properly after obtaining molecular data for *M. joannae* from its type locality in Australia.

To summarize, in this study we describe a taxon new to science, *Macrobiotus hannae* **sp. nov.**, by using an integrative approach that involved morphological and morphometric examination by PCM and SEM as well as DNA sequencing. The description of the new species questions the European records of *M. joannae* and limits its geographic distribution to Australia. Thanks to our discovery, the number of tardigrade species known from Poland has now increased to 111.

Nomenclatural acts: This work and the nomenclatural acts it contains have been registered in ZooBank. The ZooBank Life Science Identifier (LSID) for this publication is: http://zoobank.org/urn:lsid:zoobank.org:pub:74053437-C6D6-47B1-B308-B73ABDE69340

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