ZOOSYSTEMATICA ROSSICA

ISSN 2410-0226 [online] 0320-9180 [print]

Zoological Institute, Russian Academy of Sciences, St Petersburg ▪ https://www.zin.ru/journals/zsr/

Vol. 30(1): 101–115 ▪ Published online 16 June 2021 ▪ DOI 10.31610/zsr/2021.30.1.101

RESEARCH ARTICLE

Integrative description of *Hypsibius repentinus* sp. nov. (Eutardigrada: Hypsibiidae) from Sweden

Интегративное описание *Hypsibius repentinus* sp. nov. (Eutardigrada: Hypsibiidae) из Швеции

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Abstract. A new species of tardigrade from the genus *Hypsibius* Ehrenberg, 1848 is described from the bottom sediments of a small lake in the central part of Scandinavian Peninsula (Sweden), using an integrative approach, i.e. morphological techniques (light and scanning electron microscopy) combined with a molecular analysis (18S rRNA, 28S rRNA, ITS-2 and COI markers). *Hypsibius repentinus* **sp. nov.** belongs to the *Hypsibius dujardini* species–group and differs from the most similar species of this group in having a second macroplacoid with a definite constriction and in some other morphometric characters. Morphological diagnosis for the *Hypsibius dujardini* species–group is proposed, and its composition is discussed.

Резюме. Новый вид тихоходок из рода *Hypsibius* Ehrenberg, 1848 описан из донных осадков небольшого озера, находящегося в центральной части Скандинавского полуострова (Швеция). Описание выполнено с использованием методов интегративной таксономии, т.е. основано на сочетании данных световой и электронной микроскопии и изучения последовательностей нескольких молекулярно-генетических маркеров (18S rRNA, 28S rRNA, ITS-2 и COI). *Hypsibius repentinus* **sp. nov.** относится к группе видов *Hypsibius dujardini*. Новый вид отличается от наиболее близких видов группы наличием отчетливой перетяжки на втором макроплакоиде и несколькими морфометрическими признаками. Предложен морфологический диагноз группы видов *Hypsibius dujardini*; обсуждается ее состав.

Key words: freshwater tardigrades, Europe, Tardigrada, Hypsibioidea, Hypsibiidae, *Hypsibius*, *Hypsibius dujardini* species–group, new species

Ключевые слова: пресноводные тихоходки, Европа, Tardigrada, Hypsibioidea, Hypsibiidae, *Hypsibius*, группа видов *Hypsibius dujardini*, новый вид

ZooBank Article LSID: urn:lsid:zoobank.org:pub:EC8DA0BE-52DB-4F14-8AA0-5044B42CBF9F

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Introduction

The phylum Tardigrada Doyère, 1840 is a group of microscopic multicellular animals widely distributed in the nature (Nelson et al., 2018). About 1350 tardigrade species are currently known (Degma et al., 2020), but the actual number is likely to be much greater. The global diversity of tardigrades is understudied because of methodological difficulties and also because only few taxonomists are active in this field (Guil & Cabrero-Sañudo, 2007; Bartels et al., 2016). However, owing to the advances in the use of the molecular methods and to a series of recent integrative redescriptions of type species of numerous tardigrade genera (e.g. Bertolani et al., 2011; Michalczyk et al., 2012; Gąsiorek et al., 2016, 2017, 2018a; Kaczmarek et al., 2018b; Stec et al., 2018, 2020; Guidetti et al., 2019; Cesari et al., 2020; Grobys et al., 2020), increasingly more new species are being described.

Hypsibius Ehrenberg, 1848 is the type genus of the family Hypsibiidae Pilato, 1969 and, with 38 described species (Degma et al., 2020), it is the second largest genus in the family. Morphological heterogeneity within this genus as well as phylogenetic clues suggest that *Hypsibius* is a polyphyletic taxon (Guil & Giribet, 2012; Gąsiorek et al., 2018a). A combined morphological and molecular phylogenetic analysis of some species and species groups traditionally assigned to this genus revealed that they were significantly different from the "typical" *Hypsibius* species, and the genera *Borealibius* Pilato, Guidetti, Rebecchi, Lisi, Hansen et Bertolani, 2006 and *Cryobiotus* Dastych, 2019 were established. These two genera are, however, closely related to the "core" of the genus *Hypsibius*, which includes species traditionally referred to as "*dujardini* group" or "*Hypsibius dujardini* complex" (Kiehl et al., 2007; Gąsiorek et al., 2018a; Zawierucha et al., 2020) and a poorly studied "*Hypsibius convergens* complex". On the other hand, several forms initially described as belonging to *Hypsibius* proved to be phylogenetically distant not only from other members of the genus but also from the subfamily Hypsibiinae. The genera *Acutuncus* Pilato et Binda, 1997 and *Mixibius* Pilato, 1992, were shown as taking a stand-alone position within the Hypsibiidae, being not closely related to the genus *Hypsibius* (Sands et al., 2008; Marley et al., 2011; Bertolani et al., 2014; Tumanov, 2020). The genus *Notahypsibius* Tumanov, 2020 was established for the species originally described as *Hypsibius pallidoides* Pilato, Kiosya, Lisi, Inshina et Biserov, 2011 and for two morphologically similar species, because the molecular phylogenetic analysis revealed an affinity of *N. pallidoides* to the subfamily Pilatobiinae (Tumanov, 2020). A complex of species similar to *H. scabropygus* Cuénot, 1929 seems to be distinct from the "typical" *Hypsibius* species both morphologically and in the molecular phylogenetic analysis, and may have to be excluded from this genus (Zawierucha et al., 2014; Gąsiorek et al., 2018a; Tumanov, 2020).

A recent integrative redescription of the type species *Hypsibius dujardini* (Doyère, 1840) (Gąsiorek et al., 2018a) provides a solid basis for a broad investigation of the actual diversity of the "*dujardini* group". It seems to be, in fact, a large complex of closely related (semi)cryptic species widely distributed worldwide (McInnes, 1994; Kaczmarek et al., 2014, 2015, 2016; McInnes et al., 2017).

In 2017, Raquel Pereira (Uppsala University) took a sample of bottom sediments from Lake Norderåssjön, Sweden. The sample was transferred to St Petersburg by Vasily Zlatogursky and used to establish a culture of Heliozoa. Unexpectedly, a rich culture of a single tardigrade species has developed in one of the subsamples. It looked similar to *H. dujardini* but a combined morphological and molecular analysis showed that it was a new *Hypsibius* species. Its description is given in this paper.

Material and methods

Sampling and culturing

The bottom sediments of Lake Norderåssjön (Jämtland, Sweden) were collected by hand, using a small vial, in the nearshore zone. The material was later distributed over several Petri dishes. A culture of tardigrades developed in one of them, together with rotifers, protozoans, filamentous and coccoid green algae. The culture was maintained at 16 °C. For investigation, tardigrade specimens were picked up directly from the culture with a small pipette, and rinsed twice in double distilled water.

Microscopy and imaging

For light microscopy (LM), tardigrades were fixed with acetic acid or relaxed by incubating live individuals at 60 °C for 30 minutes (Morek et al., 2016) and mounted on slides in Hoyer's medium. Permanent slides were examined under a Leica DM2500 microscope equipped with phase contrast (PhC) and differential interference contrast (DIC). Photographs were taken using a Nikon DS-Fi3 digital camera with NIS software.

For scanning electron microscopy (SEM), specimens were thermally relaxed at 60 °C (Morek et al., 2016), dehydrated in an ascending ethyl alcohol series (10%, 20%, 30%, 50%, 70%, 96%) and acetone, critical-point dried in carbon dioxide, mounted on stubs and coated with gold. A Tescan MIRA3 LMU Scanning Electron Microscope was used for observations (Core Facilities Centre "Centre for Molecular and Cell Technologies", St Petersburg State University).

Morphometrics

Sample size for morphometrics was chosen following the recommendations of Stec et al. (2016). All measurements are given in micrometres (μm), followed by standard deviation values (SD). Structures were measured only if their orientations were suitable. Length of the body was measured from the anterior end to the posterior end, without hind legs. The bucco-pharyngeal tube was measured from the anterior margin of the stylet sheaths to the caudal end of the buccal tube, not including the buccal apophyses. The terminology related to the structure of the bucco-pharyngeal apparatus and the claws follows Pilato & Binda (2010). Elements of the buccal apparatus were measured according to Kaczmarek & Michalczyk (2017). Claws were measured following Beasley et al. (2008), except that their total length was also measured (according to Pilato et al., 2002). The *pt* index, which is the percentage ratio between the length of a structure and the length of the buccal tube (Pilato, 1981), is presented here in italics. Morphometric data were treated using ver. 1.6 of the "Parachela" template available from the Tardigrada Register (Michalczyk & Kaczmarek, 2013), with addition of the total length of the claws.

Genotyping

DNA was extracted from two specimens with use of QuickExtract™ DNA Extraction Solution (Lucigen Corporation, USA) using the protocol kindly provided by Torbjørn Ekrem, Norwegian University of Science and Technology (for complete protocol description see Tumanov, 2020). The exoskeletons were recovered, mounted on a microscope slides in Hoyer's medium and retained as the hologenophores (Pleijel et al., 2008).

Four genes were sequenced from both specimens: a small ribosome subunit (18S rRNA) gene, a large ribosome subunit (28S rRNA) gene, an internal transcribed spacer (ITS-2), and the cytochrome oxidase subunit I (COI) gene. The primers and PCR programs used are provided in Table 1.

The PCR products were visualised in 1.5% agarose gel stained with ethidium bromide. All amplicons were sequenced directly using the ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) using an ABI Prism 310 Genetic Analyser in "Centre for Molecular and Cell Technologies" of St Petersburg State University. The sequences were edited and assembled using ChromasPro software (Technelysium, USA). The COI sequences were translated to amino acids using the invertebrate mitochondrial code, implemented in MEGA7 (Kumar et al., 2016), in order to check for the presence of stop codons and therefore of pseudogenes.

All sequences of the subfamily Hypsibiinae (the genera *Borealibius*, *Cryobiotus* and *Hypsibius*) available in GenBank (www.ncbi.nlm.nih.gov/genbank/) at the time of the analysis were downloaded, and those originating from published works with a reliable attribution of the investigated taxa were selected (see Electronic supplementary material 1). The sequences were aligned using the Muscle algorithm (Edgar, 2004) with default settings, as implemented in SeaView ver. 4.0 (Gouy et al., 2010). Uncorrected pairwise distances were calculated using MEGA7 (Kumar et al., 2016) with gaps/missing data treatment set to "complete deletion". All the obtained sequences were deposited in GenBank.

Results

Phylum **Tardigrada** Doyère, 1840

Class **Eutardigrada** Richters, 1926

Superfamily **Hypsibioidea** Pilato, 1969

Family **Hypsibiidae** Pilato, 1969

Subfamily **Hypsibiinae** Pilato, 1969

Genus ***Hypsibius*** Ehrenberg, 1848

***Hypsibius repentinus* sp. nov.**
(Figs 1–4)

Holotype. Female; **Sweden**, *Härjedalen*, approx. 62°8′52″N 13°57′23″E, ca. 400 m a.s.l., Lake Norderåssjön, bottom near shore, plant debris, 30 Aug. 2017, Raquel Pereira leg. (SPbU 254(32)).

Paratypes. 42 females, 2 exuviae with eggs, 10 cysts, same data as for holotype (SPbU 254(1) to 254(31) and 254(33) to 254(35)); 5 adults, 15 cysts, same data as for holotype, SEM stub (SPbU_Tar20). All the type specimens are kept at St Petersburg State University.

Morphological description. Body elongate, slightly widened in the region of legs III (Fig. 1), with a blunt snout (morphometrics: Table 2 and Electronic supplementary material 2).

Colour. Body transparent or whitish. Live specimens with black eyespots, becoming invisible after mounting in Hoyer's medium (Fig. 1A).

Cuticular sculpture. Cuticle looking smooth under LM; rugose sculpture better developed dorsally, visible over body surface under SEM (Figs 1B–D, 2A, B). No external cephalic sensory structures (Fig. 2B).

Mouth. Opening anteroventral, on developed mouth cone; mouth opening surrounded by simple cuticular fold, without distinct peribuccal lobes or papulae (Fig. 2C). In some specimens, a line of elliptical structures visible around mouth opening under LM (Fig. 2F: black arrowhead), similar to those described in *Notahypsibius pallidoides*, *Acutuncus antarcticus* (Richters, 1904) and *Hypsibius murrayi* (Richters, 1907) (Dastych, 1991, 2018; Tumanov, 2020).

Bucco-pharyngeal apparatus of Hypsibiinae model (Fig. 2D). Oral cavity armature with a ring of small teeth located in anterior part (visible under SEM only; Fig. 2C, E). Dorsal and ventral apophyses for insertion of stylet muscles similar in shape and size (Fig. 2G, H). Buccal tube rigid, often slightly bent ventrally in caudal part. Stylet furcae typically-shaped. Pharyngeal bulb spherical, with well-developed apophyses, two elongate macroplacoids and large septulum (Fig. 2D, I, K). No microplacoids or pseudoseptulum.

First macroplacoid slightly longer than second; both macroplacoids with distinct (well discernible

Table 1. Primers and PCR programs used for amplification of the four DNA fragments sequenced in the study.

DNA fragment	Primer name	Primer direction	Primer sequence (5'–3')	Primer source	PCR programme
COI	LCO1490	forward	GGTCAACAAATCATAAAGATATTGG	Folmer et al., 1994	Michalczyk et al., 2012
	HCO2198	reverse	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al., 1994	
	COI_Para_F*	forward	TTTCAACAAACCACAAAGATATYGG	Gąsiorek et al., 2018a	
	COI_Eutar_Rr	reverse	TAAACTTCTGGGTGACCRAARAAYCA	Gąsiorek et al., 2018a	
18S rRNA	18S_Tar_Ff1	forward	AGGCGAAACCGCGAATGGCTC	Stec et al., 2017	Stec et al., 2015 (from the unpublished thesis by Zeller, 2010)
	18S_Tar_Rr1	reverse	GCCGCAGGCTCCACTCCTGG	Stec et al., 2017	
	SSU_F_04	forward	GCTTGTCTCAAAGATTAAGCC	Kiehl et al., 2007	
	SSU_R_26	reverse	CATTCTTGGCAAATGCTTTCG	Kiehl et al., 2007	
28S rRNA	28S_Eutar_F	forward	ACCCGCTGAACTTAAGCATAT	Gąsiorek et al., 2018b	Mironov et al., 2012
	28S_R0990	reverse	CCTTGGTCCGTGTTTCAAGAC	Mironov et al., 2012	
ITS-2	ITS2_Eutar_Ff	forward	CGTAACGTGAATTGCAGGAC	Stec et al., 2018	Stec et al., 2018
	ITS2_Eutar_Rr	reverse	TGATATGCTTAAGTTCAGCGG	Stec et al., 2018	

* Under this name, the sequence of Folmer's LCO1490 primer was erroneously given in Gąsiorek et al. (2018). The correct sequence of the primer was received from P. Gąsiorek (pers. comm.).

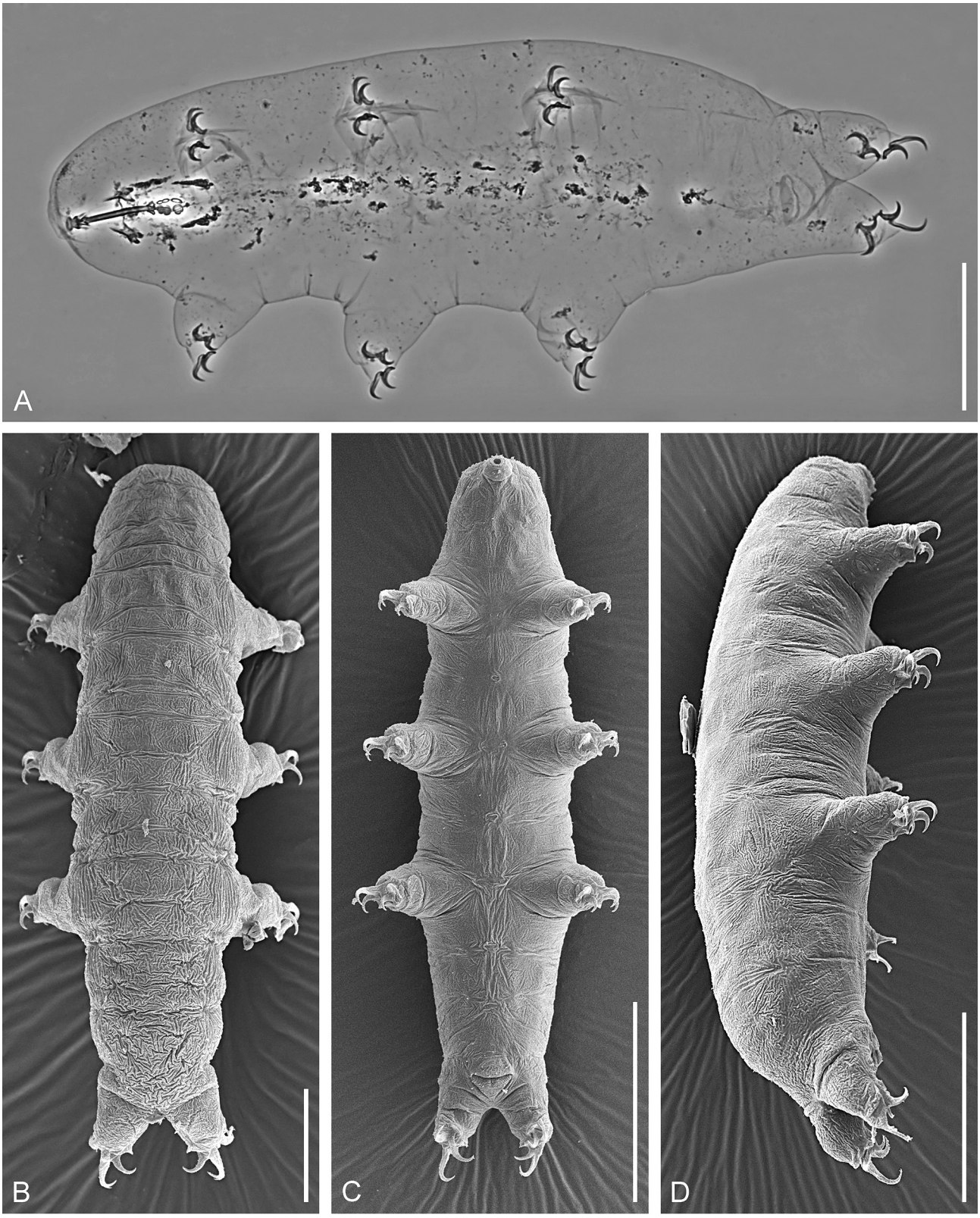
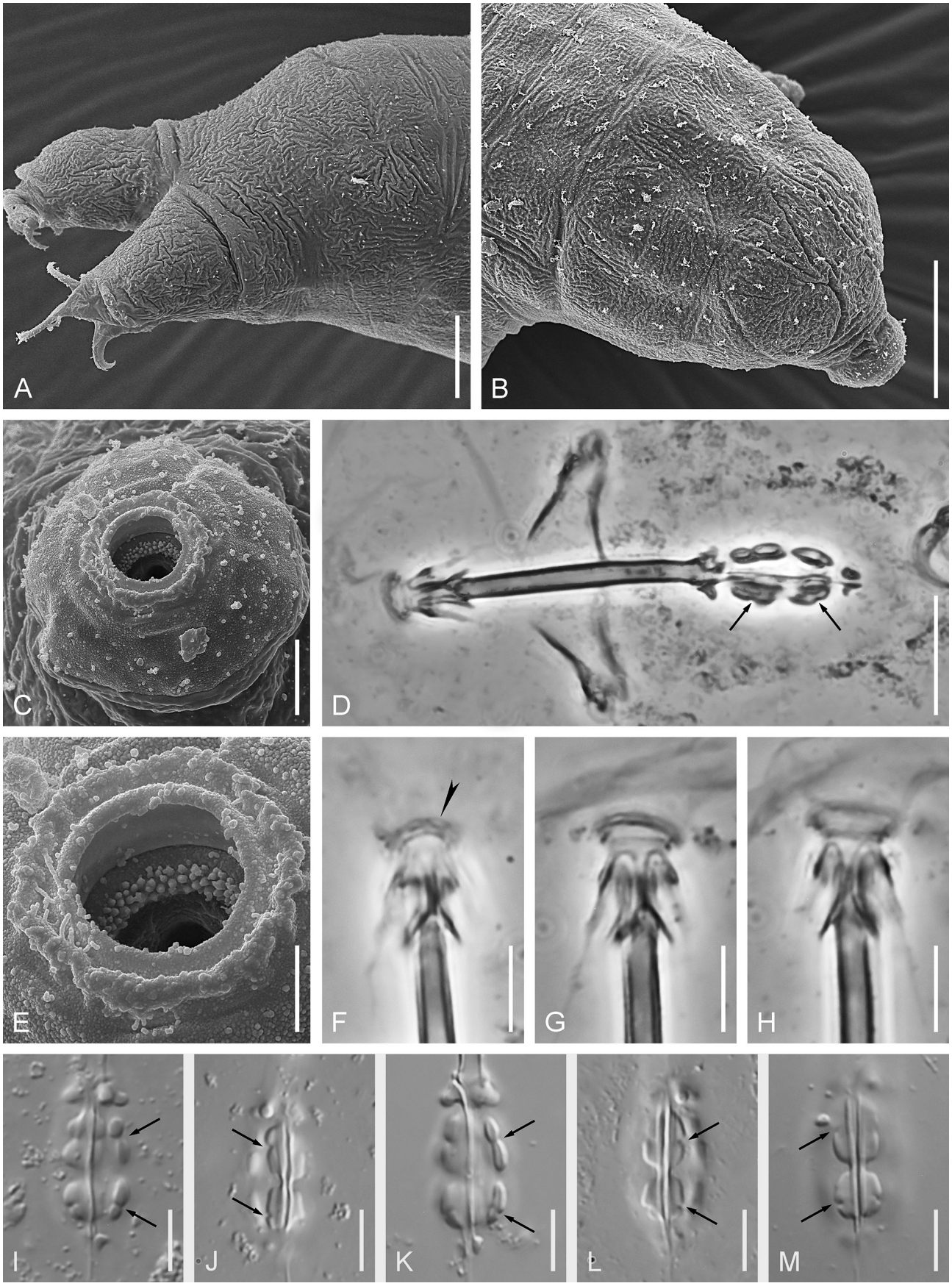


Fig. 1. *Hypsibius repentinus* **sp. nov.**, habitus. **A**, general dorsoventral view (PhC, holotype); **B**, dorsal view (SEM); **C**, ventral view (SEM); **D**, lateral view (SEM). Scale bars: A, C, D – 50 μm, B – 20 μm.


A
B
C
D
E
F
G
H
I
J
K
Ł
M

under LM) constrictions: in the middle of first macroplacoid and slightly closer to caudal part of second macroplacoid (Fig. 2D, I–M: black arrows).

Legs and claws. All legs with well-developed claws, increasing in size from legs I to IV (Figs 1A, 3A–F). Claws of *Hypsibius*-type with external and internal claws of each leg evidently dissimilar. All claws with developed accessory points, free apices of accessory points of external claws I–III usually positioned lateral to main branch and poorly discernible under LM (Fig. 3A, E). Bases of all claws smooth, slightly widened. Claws of legs I–III with poorly developed smooth lunules (= pseudolunules, according to Gąsiorek et al., 2017) (Fig. 3B: black arrows). Claws of legs IV with more developed lunules (usually larger and better visible at bases of anterior claws; Fig. 3D: black arrow) and a cuticular bar between the bases of the anterior and the posterior claws (Fig. 3C, D, black arrowheads). Legs I–III without cuticular bars near claw bases but with an elongated bulge located near base of internal claw (visible under SEM only; Fig. 3E: white arrowhead).

Cysts. Numerous cysts were observed in the culture on the late stage of its development. Cysts oval (mean length 113.2 ± 10.3 μm, mean width 69.9 ± 3.3 μm; n = 10), with thickened cuticle forming more or less developed circular transverse folds (Fig. 4A–C). Legs contracted, not raised above body surface (Fig. 4A, C), with strongly modified claws being shorter and much more massive than claws of active forms (Fig. 4D, E). No additional layers of cuticle visible below cyst wall.

Eggs. One to four white subspherical eggs with smooth shell laid in exuviae, 58.0–63.0 μm in diameter (mean 60.6 ± 2.3 μm; n = 5).

Reproduction mode. No males were observed, which suggest a parthenogenetic type of reproduction.

Phenotypic comparison. *Hypsibius repentinus* **sp. nov.** belongs to the *Hypsibius dujardini* species–group, recognised here as a group of species with a smooth or slightly rugose cuticle, two macroplacoids and a septulum (or microplacoid) in the pharynx (see Discussion). This group currently contains eleven species: *H. allisoni* Horning, Schuster et Grigarick, 1978, *H. conwentzii* Kaczmarek, Parnikoza, Gawlak, Esefeld, Peter, Kozeretska et Roszkowska, 2018, *H. dujardini*, *H. exemplaris* Gąsiorek, Stec, Morek et Michalczyk, 2018, *H. iskandarovi* Tumanov, 1997, *H. murrayi*, *H. pachyunguis* Maucci, 1996, *H. septulatus* Pilato, Binda, Napolitano et Moncada, 2004, *H. seychellensis* Pilato, Binda et Lisi, 2006, *H. valentinae* Pilato, Kiosya, Lisi et Sabella, 2012, and *H. vaskelae* Tumanov, 2018.

Hypsibius repentinus **sp. nov.** clearly differs from *H. conwentzii*, *H. iskandarovi*, *H. murrayi*, *H. septulatus* and *H. vaskelae* in the absence of cuticular bars on legs I–III, and from *H. allisoni* and *H. pachyunguis*, in having a large distinct septulum instead of small dot-like structures usually considered as microplacoids (Horning et al., 1978; Pilato et al., 2012; Tumanov, 2018).

Hypsibius repentinus **sp. nov.** differs from *H. seychellensis* in having a wider buccal tube (*pt* value for the external buccal tube width 7.9–10.9 in *H. repentinus* **sp. nov.** *vs.* 6.3–6.4 in *H. seychellensis*) and in the presence of a constriction of the second macroplacoid visible under LM.

Hypsibius repentinus **sp. nov.** differs from *H. valentinae* in having a slightly wider buccal tube (*pt* value for the external buccal tube width 7.9–10.9 in *H. repentinus* **sp. nov.** *vs.* 7.4–7.8 in *H. valentinae*), the higher *pt* value for the stylet support inserting point (62.8–66.8 in *H. repentinus* **sp. nov.** *vs.* 61.3–62.5 in *H. valentinae*), the shorter external claws of legs II (*pt* value 41.6–56.3 in *H. repentinus* **sp. nov.** *vs.* 60.2–60.3 in *H. valentinae*) and in the presence of a constriction of the second macroplacoid visible under LM.

Hypsibius repentinus **sp. nov.** is very similar to *H. dujardini* and *H. exemplaris*, differing from them only in the presence of a constriction of the second macroplacoid visible under LM.

Fig. 2. *Hypsibius repentinus* **sp. nov.**, body surface and bucco-pharyngeal apparatus. **A**, caudal part of the body, laterodorsal view (SEM); **B**, cephalic part of the body, laterodorsal view (SEM); **C**, mouth cone (SEM); **D**, bucco-pharyngeal apparatus (PhC); **E**, mouth opening and oral cavity (SEM); **F**, anterior margin of the mouth opening (PhC); **G**, dorsal apophyses for insertion of stylet muscles (PhC, holotype); **H**, ventral apophyses for insertion of stylet muscles (PhC, holotype); **I–M**, placoids of different specimens (DIC). Black arrows indicate the constrictions on the placoids, black arrowhead indicates the elliptical structures. Scale bars: A, B, D – 10 μm, C – 2 μm, E – 1 μm, F–M – 5 μm.

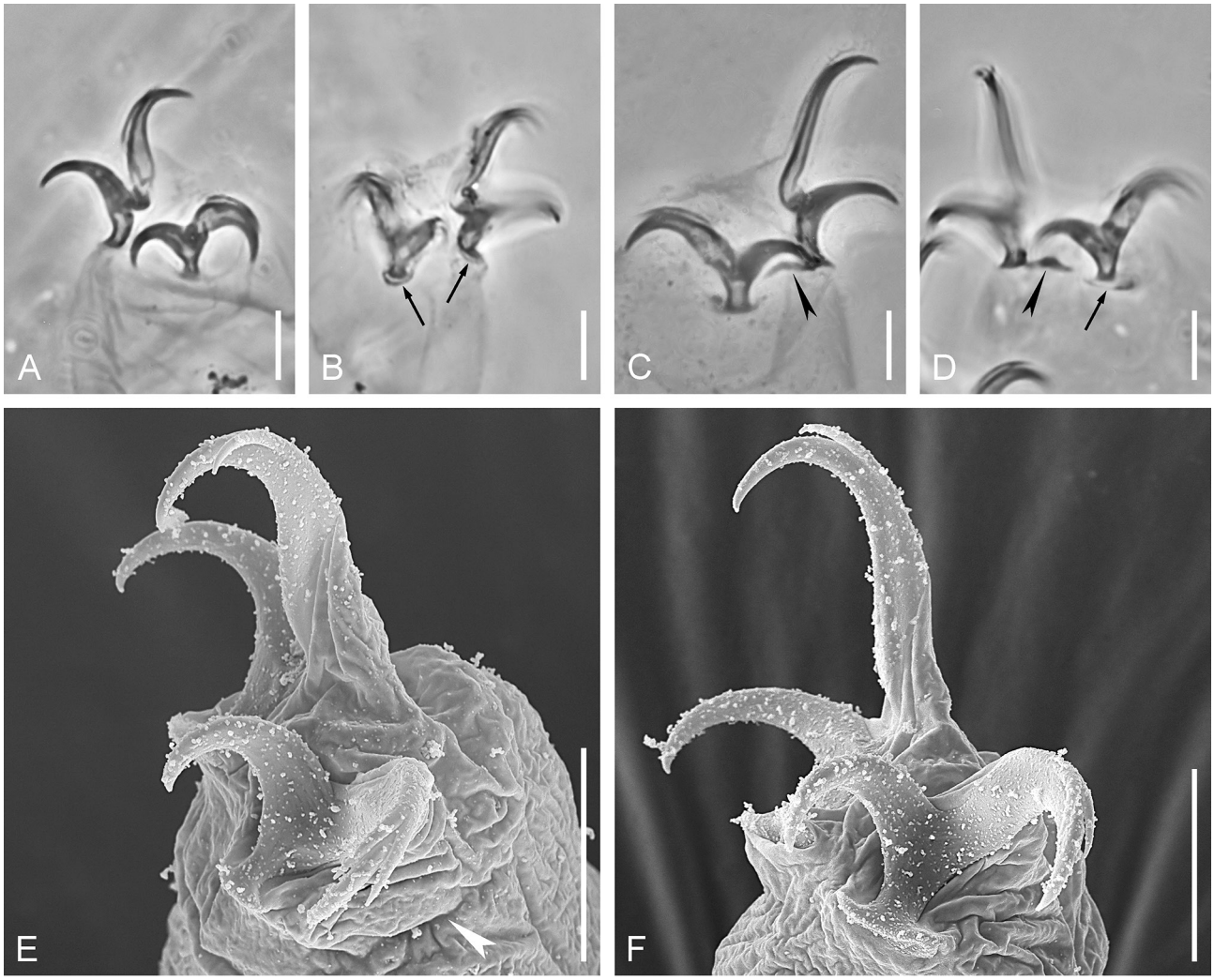


Fig. 3. *Hypsibius repentinus* **sp. nov.**, claws. **A**, claws I (PhC); **B**, lunules on claws I (PhC); **C**, **D**, claws IV (PhC); **E**, claws II (SEM); **F**, claws IV (SEM). Black arrows indicate the lunules; black arrowheads indicate the cuticular bar; white arrowhead indicates the bulge. Scale bars: 5 μm.

It should be noted that a form with a very similar configuration of the buccal-pharyngeal apparatus, with an evident constriction on the second macroplacoid, was briefly described and illustrated from Italy by Bertolani (1982) as belonging to *H. dujardini*. As with other numerous records of *H. dujardini* published prior to the redescription of this species (Gąsiorek et al., 2018a), the real taxonomic status of this form is unclear, but it can be an evidence of the presence of *H. repentinus* in Italy.

DNA sequences. Sequences of good quality for the four molecular markers mentioned above were obtained from the two hologenophores: voucher specimens 254(17) and 254(18). Each gene was represented by a single haplotype. All obtained sequences were deposited in GenBank.

COI sequence (GenBank: MW549048 and MW549049), 645 and 648 bp long.

18S rRNA sequence (GenBank: MN927183 and MN927184), 852 and 830 bp long.

28S rRNA sequence (GenBank: MW549063 and MW549064), 800 and 802 bp long.

ITS-2 sequence (GenBank: MW549061 and MW549062), 457 and 469 bp long.

Molecular comparison. The ranges of uncorrected genetic *p*-distances between the studied population of *Hypsibius repentinus* **sp. nov.** and the species of the subfamily Hypsibiinae whose sequences are available from GenBank

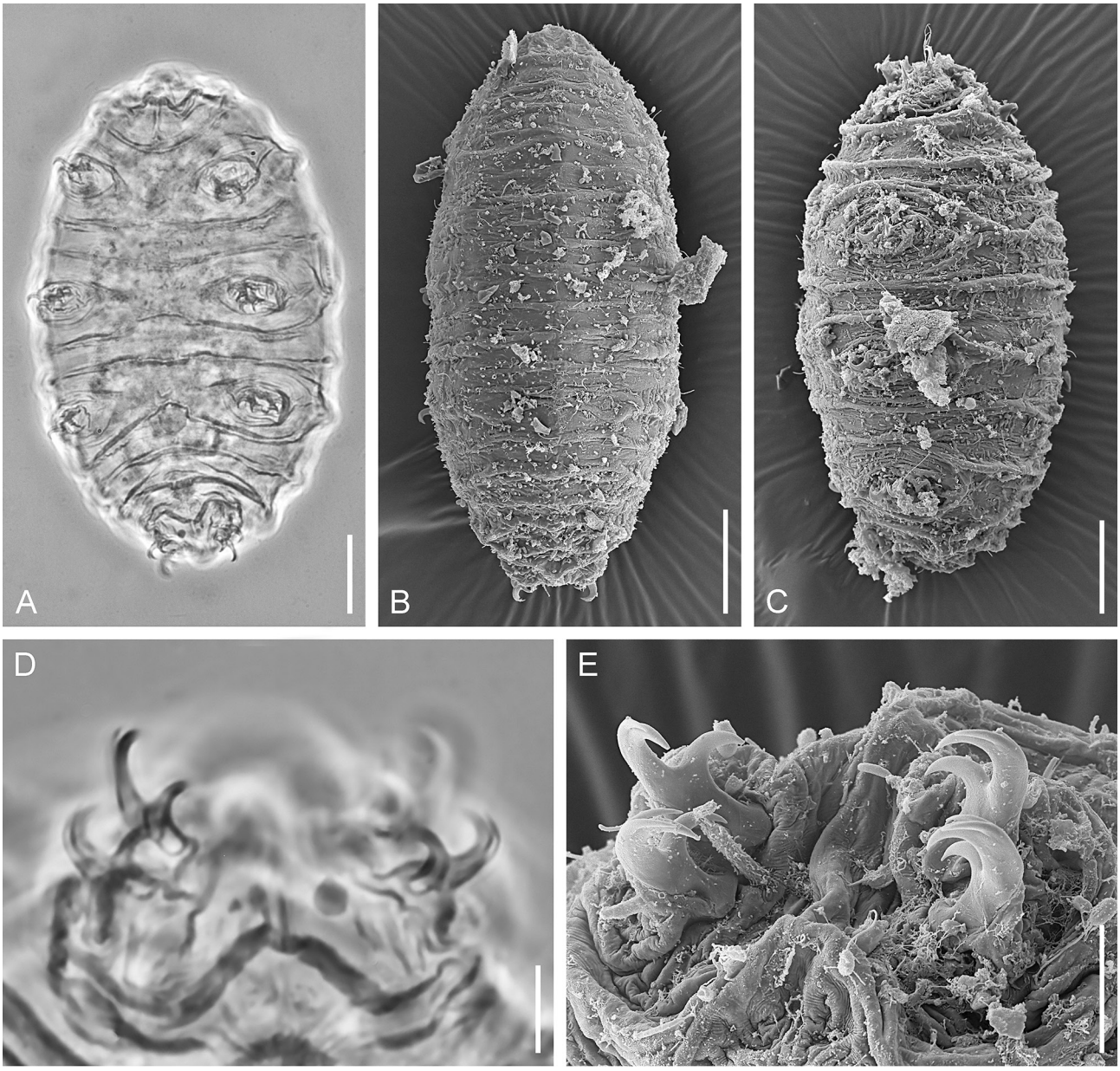


Fig. 4. *Hypsibius repentinus* **sp. nov.**, cysts. **A**, ventral view (PhC); **B**, dorsal view (SEM); **C**, lateral view (SEM); **D**, claws IV (PhC); **E**, claws IV (SEM). Scale bars: A–C – 20 μm; D, E – 5 μm.

(see Electronic supplementary material 1) are as follows:

COI: 17.4%–24.5% (mean 20.5%), with the most similar being *Hypsibius* cf. *exemplaris* 3 (MW010376: Zawierucha et al., 2020) and the least similar being *Cryobiotus klebelsbergi* (Mihelčič, 1959) (KT901831: Dabert et al., 2015).

18S rRNA: 0.0%–4.1% (mean 2.0%), with the most similar being *H. exemplaris* (HQ604943: Gąsiorek et al., 2018a) and *Hypsibius* sp. (EU266939: Sands et al., 2008) and the least similar being *H. scabropygus* (KC582831: Dabert et al., 2014).

28S rRNA: 0.5%–3.2% (mean 2.2%), with the most similar being *H. exemplaris* (MG800337: Gąsiorek et al., 2018a) and the least similar being *C. klebelsbergi* (KC582835: Dabert et al., 2014).

ITS-2: 2.8%–11.6% (mean 7.2%), with the most similar being *H. exemplaris* (MG800336: Gąsiorek et al., 2018a) and the least similar being *H. dujardini* (MG777531: Gąsiorek et al., 2018a).

Table 2. Summary of morphometric data for *Hypsibius repentinus* **sp. nov.** Measurements are given in μm, *pt* values in % (*pt* index is percentage ratio between the length of a structure and the length of the buccal tube).

Character	n	Range		Mean		SD		Holotype	
		μm	*pt*	μm	*pt*	μm	*pt*	μm	*pt*
Body length	30	142–269	*571–1015*	227	*915*	26	*86*	269	*998*
Buccopharyngeal tube									
Buccal tube length	30	21.4–27.4	–	24.8	–	1.4	–	27.0	–
Stylet support insertion point	30	13.5–17.8	*62.8–66.8*	16.0	*64.6*	1.0	*1.0*	17.5	*65.1*
Buccal tube external width	30	1.9–2.6	*7.9–10.9*	2.2	*9.0*	0.2	*0.9*	2.2	*8.2*
Buccal tube internal width	30	0.9–1.5	*3.6–5.6*	1.1	*4.5*	0.2	*0.5*	1.4	*5.2*
Placoid lengths									
Macroplacoid 1	30	3.3–5.0	*13.5–21.2*	4.3	*17.5*	0.4	*1.8*	4.4	*16.2*
Macroplacoid 2	30	3.1–4.2	*12.5–17.0*	3.6	*14.4*	0.3	*1.2*	3.8	*14.0*
Septulum	30	0.9–1.7	*3.5–7.4*	1.2	*5.0*	0.2	*1.0*	1.2	*4.3*
Macroplacoid row	30	7.2–9.8	*29.1–41.2*	8.5	*34.3*	0.6	*2.7*	8.6	*31.8*
Placoid row (incl. septulum)	30	9.0–11.7	*37.1–54.3*	10.8	*43.5*	0.7	*3.7*	11.1	*41.0*
Claw 1 lengths									
External base	12	3.5–4.8	*15.3–18.7*	4.2	*17.1*	0.4	*1.1*	4.8	*17.9*
External primary branch	13	6.2–9.5	*26.7–34.6*	7.5	*30.2*	1.0	*2.9*	7.9	*29.2*
External secondary branch	13	4.8–6.8	*20.1–27.2*	5.7	*23.0*	0.7	*2.0*	5.9	*21.7*
External total	11	9.3–13.5	*40.8–51.7*	11.3	*45.8*	1.3	*3.5*	11.8	*43.7*
Internal base	13	3.2–4.2	*13.5–17.1*	3.7	*14.7*	0.3	*1.1*	3.7	*13.7*
Internal primary branch	13	4.6–6.2	*19.8–24.6*	5.6	*22.3*	0.5	*1.4*	6.2	*22.9*
Internal secondary branch	11	3.4–5.2	*14.8–20.7*	4.4	*17.4*	0.6	*1.8*	4.8	*17.9*
Internal total	13	6.7–8.2	*26.9–34.6*	7.4	*29.8*	0.5	*2.3*	7.4	*27.5*
Claw 2 lengths									
External base	14	3.7–5.3	*15.0–19.5*	4.3	*17.5*	0.4	*1.6*	5.3	*19.5*
External primary branch	14	6.0–9.6	*26.5–37.5*	8.2	*33.1*	1.0	*3.6*	8.4	*31.2*
External secondary branch	13	4.8–6.8	*19.0–27.2*	5.8	*23.2*	0.6	*2.2*	6.6	*24.6*
External total	14	9.5–13.3	*41.6–56.3*	12.0	*48.6*	1.1	*4.0*	12.6	*46.7*
Internal base	14	3.1–4.2	*12.5–17.2*	3.7	*14.9*	0.3	*1.5*	4.1	*15.1*
Internal primary branch	14	5.8–7.6	*23.5–29.5*	6.7	*26.6*	0.5	*1.6*	7.2	*26.7*
Internal secondary branch	13	4.1–5.7	*17.7–23.1*	4.9	*19.4*	0.5	*1.4*	5.5	*20.2*
Internal total	14	7.1–9.2	*28.3–37.3*	8.1	*32.6*	0.7	*2.7*	8.5	*31.6*
Claw 3 lengths									
External base	11	3.9–5.7	*14.9–22.0*	4.6	*18.1*	0.6	*1.9*	5.1	*19.1*
External primary branch	11	5.8–9.2	*23.4–35.2*	7.4	*29.6*	1.1	*3.5*	8.4	*31.1*
External secondary branch	11	4.9–8.0	*21.6–29.5*	5.9	*23.5*	0.8	*2.5*	5.8	*21.6*
External total	8	10.1–13.6	*41.7–51.3*	11.9	*46.5*	1.1	*3.2*	12.7	*47.2*
Internal base	9	3.3–4.2	*12.7–16.3*	3.6	*14.7*	0.3	*1.1*	3.4	*12.7*
Internal primary branch	9	5.9–7.3	*24.5–30.1*	6.8	*27.5*	0.5	*1.9*	6.8	*25.1*
Internal secondary branch	8	4.0–5.7	*17.7–21.6*	5.0	*20.2*	0.6	*1.3*	5.7	*21.1*
Internal total	8	7.0–8.9	*28.5–35.8*	7.9	*32.7*	0.6	*2.3*	?	*?*
Claw 4 lengths									
Anterior base	22	3.2–5.3	*13.6–19.6*	4.2	*16.7*	0.5	*1.7*	5.0	*18.6*
Anterior primary branch	22	5.8–9.0	*23.7–35.1*	7.6	*30.7*	0.8	*2.4*	9.0	*33.4*
Anterior secondary branch	21	3.8–6.2	*16.6–24.9*	5.1	*20.6*	0.6	*2.1*	5.9	*21.7*
Anterior total	22	8.6–11.8	*36.1–43.7*	10.0	*40.0*	0.8	*1.7*	11.8	*43.7*
Posterior base	26	3.8–5.8	*15.3–22.7*	4.8	*19.2*	0.6	*1.9*	5.1	*19.1*
Posterior primary branch	26	8.0–11.5	*33.4–43.9*	9.9	*39.7*	1.0	*3.2*	11.5	*42.7*
Posterior secondary branch	26	5.4–7.7	*21.9–29.1*	6.4	*25.8*	0.6	*1.9*	6.7	*24.8*
Posterior total	25	11.4–16.4	*46.5–62.7*	14.1	*56.7*	1.4	*4.8*	16.4	*61.0*

Full matrices with *p*-distances are provided in Electronic supplementary material 3.

Etymology. The species epithet *repentinus* is a Latin adjective, which means "unexpected". It refers to an unexpected appearance of the species in the material prepared for culturing protists.

Discussion

There is some uncertainty in the definition and therefore in the composition of the *Hypsibius dujardini* group of species in the literature. Miller et al. (2005) defined this group as including all colourless species with a smooth cuticle and an oval pharynx with two rod-shaped macroplacoids. As the result, species devoid of microplacoids or septulum were included in the *H. dujardini* group, which is in obvious contradiction with the traditional understanding of its composition. Recently, Gąsiorek et al. (2018a) gave a different diagnosis for the *H. dujardini* group as a group of *Hypsibius* species which is characterised by smooth cuticle and two macroplacoids and septulum in the pharynx. Such definition matches the traditional understanding of the complex of species similar to *H. dujardini* (Pilato et al., 2012; Dastych, 2018; Kaczmarek et al., 2018a).

In our opinion, new descriptions and amendments of the diagnoses of several "old" *Hypsibius* species revealed the necessity of some corrections to the diagnosis of the *Hypsibius dujardini* group. First, the descriptions of *H. vaskelae* (Tumanov, 2018) and *H. repentinus* **sp. nov.** have demonstrated the presence of rugose sculpture, which can be invisible or very poorly visible under LM. Hence, the demarcation of "smooth" and "sculptured" species became unclear.

Second, there are several *Hypsibius* species with tiny cuticular structures in the pharynx located behind the macroplacoids (*H. allisoni*, *H. murrayi* and *H. pachyunguis*). Those structures could not be unequivocally interpreted as microplacoids or septulum, though are predominantly mentioned as microplacoids in the recent publications (Pilato et al., 2012; Dastych, 2018; Tumanov, 2018). Until the homology of these structures is specified, presence of the microplacoids cannot be excluded for species of the *Hypsibius dujardini* group.

Summing up, the *Hypsibius dujardini* group is recognised here as a group of *Hypsibius* species with a smooth or slightly rugose (but not granulate or tuberculate) cuticle, two macroplacoids and a septulum (or microplacoid) in the pharynx.

Phylogenetic relationships within the subfamily Hypsibiinae are poorly resolved yet, mainly because the sequences of the phylogenetically significant genes are known only for a few species. A comparison of the three best characterised species of the *H. dujardini* group (*H. dujardini*, *H. exemplaris* and *H. repentinus* **sp. nov.**) demonstrates that the truly freshwater species *H. exemplaris* and *H. repentinus* **sp. nov.** seem to be more closely related to each other than to the semiterrestrial *H. dujardini*. However, new data from a wide set of *Hypsibius* species belonging to different morphological and ecological groups are needed to verify this assumption.

Addenda

Electronic supplementary material 1. Complete list of sequences used for molecular comparisons between *Hypsibius repentinus* **sp. nov.** and all other species of the subfamily Hypsibiinae for which homologous DNA sequences are currently available. File format: PDF. Available from: https://doi.org/10.31610/zsr/2021.30.1.101

Electronic supplementary material 2. Raw morphometric data for *Hypsibius repentinus* **sp. nov.** File format: XLSX. Available from: https://doi.org/10.31610/zsr/2021.30.1.101

Electronic supplementary material 3. Matrices of *p*-distances for species of Hypsibiinae. File format: XLSX. Available from: https://doi.org/10.31610/zsr/2021.30.1.101

Acknowledgements

We would like to thank Raquel Pereira (Uppsala University) for collecting the material from which the new species was described. We are grateful to Vasily Zlatogursky (St Petersburg State University), who recognised the tardigrade culture and placed it at our disposal. We thank Torbjørn Ekrem (Norwegian University of Science and Technology) for providing an unpublished DNA extraction protocol. We extend our thanks to Piotr Gąsiorek and Łukasz Kaczmarek for their valuable comments and improvements made for the manuscript as reviewers, and to Natalia Lentsman for the linguistic review of the manuscript. This study was carried out with the use of equipment of the Core Facilities Centre "Centre for Molecular and Cell Technologies" of St Petersburg State University.

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Received 26 February 2021 / Accepted 14 June 2021. Editorial responsibility: M.E. Daneliya