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AN INTEGRATIVE DESCRIPTION OF A NEW *RICHTERSIUS* SPECIES FROM GREECE (TARDIGRADA: EUTARDIGRADA: RICHTERSIUSIDAE)

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In this paper, we describe a new tardigrade species, *Richtersius tertius* sp. n., from Greece. The description is based on morphological and morphometric analysis using light and scanning electron microscopy as well as genetic analysis based on four molecular markers (DNA sequences of three nuclear, i.e., 18S rRNA, 28S rRNA, ITS-2 and one mitochondrial COI fragment). Morphological and morphometric differences, together with genetic comparisons, provide independent verifications of *Richtersius tertius* sp. n. as a species new to science. Phenotypically, the new taxon differs from *Richtersius coronifer* (Richters, 1903) and *Richtersius ziemowiti* Kayastha, Berdi, Miaduchowska, Gawlak, Łukasiewicz, Gołdyn, Jędrzejewski et Kaczmarek, 2020 mainly by the morphology and size of cuticular pores, present only in hatchlings (first instars), as well as some morphometric characters. The results presented herein contribute further to the recognition of the morphological variability and biodiversity within *Richtersius*, with *Richtersius tertius* sp. n. being the third species formally described within the genus.

Key words: biodiversity, integrative taxonomy, *Richtersius tertius* sp. n., systematics.

INTRODUCTION

Water bears (Tardigrada), also known as moss piglets, are a globally distributed phylum of microscopic metazoans related to arthropods and onychophorans that inhabit terrestrial, marine and freshwater environments (Campbell *et al.* 2011, Nelson *et al.* 2015). To date, over 1300 species of tardigrades have been found and described (Guidetti & Bertolani 2005, Degma & Guidetti 2007, Degma *et al.* 2009–2021). Although tardigrade taxonomy is challenging because of their microscopic size and a small number of taxonomically informative characters (Kosztyła *et al.* 2016, Morek *et al.* 2016), over a dozen species new to science are described every year, further expanding our knowledge on their biodiversity (Michalczyk & Kaczmarek 2013).

Richtersius coronifer (Richters, 1903), originally described as *Macrobiotus coronifer* Richters, 1903, is characterised by large body size (up to a 1 mm in length), yellowish colour, mouth opening surrounded with a circular velum,

beak-shaped teeth of the third band in the oral cavity, thick-walled buccal tube with a narrow light, pharynx containing macroplacoids but not microplacoids, and claws on all legs equipped with strongly and regularly dentate lunulea. Since there was no type material of *M. coronifer*, Maucci and Ramazzotti (1981) redescribed the species and simultaneously transferred it to the newly erected genus *Adorybiotus* Maucci et Ramazzotti, 1981. However, the detailed morphological comparison with *Adorybiotus granulatus* (Richters, 1903) and *Adorybiotus coronifer* (Richters, 1903) by Pilato and Binda (1987) allowed the taxon to be redefined and established as the type species for a new monotypic genus *Richtersia* Pilato et Binda, 1987. After two years, due to the homonymy of the proposed name with a generic name within the Nematoda, the genus name was changed once again, from *Richtersia* to *Richtersius* by Pilato and Binda (1989). For many years *Richtersius* remained monotypic, containing only *R. coronifer*, a species considered to have a cosmopolitan distribution (McInnes 1994, Kaczmarek *et al*. 2015, Kaczmarek *et al*. 2016, McInnes *et al*. 2017). A century after it was described, the first analyses of molecular data obtained for *R. coronifer* indicated that there were more than one species hidden within the genus *Richtersius* (Rebecchi *et al.* 2003, Faurby *et al.* 2008). These results were later confirmed by Guidetti *et al*. (2016), who, based on morphological and genetic analyses, established the family Richtersiidae Guidetti, Rebecchi, Bertolani, Jönsson, Kristensen et Cesari, 2016. The new family included three genera: *Richtersius*, *Adorybiotus* and the then newly erected *Diaforobiotus* Guidetti, Rebecchi, Bertolani, Jönsson, Kristensen et Cesari, 2016, that comprises species of the former informal *Macrobiotus islandicus* group. The phylogenetic relationships within the proposed family were unclear since *Adorybiotus* was more closely related to the family Murrayidae Guidetti, Gandolfi, Rossi et Bertolani, 2005 than to the other richtersiid genera. Nevertheless, *Adorybiotus* was tentatively included within Richtersiidae because of morphological similarities with *Richtersius* and *Diaforobiotus* (Guidetti *et al.* 2016). The research mentioned above, dealing with the genus *Richtersius*, clearly indicates that global records of "*R. coronifer*" are likely to represent a species complex rather than a single ubiquitous species (Rebecchi *et al.* 2003, Faurby *et al.* 2008, Guidetti *et al.* 2016, Stec *et al.* 2020*a*). To allow a formal description of species within the complex, Stec *et al.* (2020*a*) redescribed *R. coronifer* using a population collected from one of the two localities in Spitsbergen mentioned in the original description. To secure and stabilise the taxonomy of the genus *Richtersius* an integrative approach to the redescription was adopted and linked with setting aside the neotype designated by Maucci and Ramazzotti (1981) and with the designation of the new neotype (Stec *et al*. 2020*a*, Stec & Michalczyk 2020). The effects of the redescription are already noticeable, as recently a second species in the genus, *Richtersius ziemowiti* Kayastha, Berdi, Mioduchowska, Gawlak, Łukasiewicz, Gołdyn,

Jędrzejewski et Kaczmarek, 2020, was described from Nepal (previously reported as *R. coronifer* by Kayastha *et al*. 2020*b*).

In addition to the redescription of *R. coronifer* published at the beginning of 2020, the year was an intense period regarding changes made within the systematics of the family Richtersiidae. Specifically, the new genus *Crenubiotus* Lisi, Londoño et Quiroga, 2020 was erected using classical taxonomic methods and detailed morphological examination of the animals and eggs of *Macrobiotus crenulatus* (Richters, 1904) as well as those of a very similar new species from Colombia, and then placing the new genus within the family Richtersiidae. Subsequently, Stec *et al.* (2020*b*) integratively redescribed *Crenubiotus crenulatus,* pinpointing its phylogenetic position within Macrobiotoidea. The analysis, in addition to the new genetic data for two analysed *Crenubiotus* species, also included novel genetic data for *Adorybiotus* cf. *granulatus,* demonstrating the presence of a distinct evolutionary lineage composed of *Adorybiotus* and *Crenubiotus*. In order to acknowledge the phylogenetic and morphological distinctiveness of this lineage, (Stec *et al.* 2020*b*) erected a new family, Adorybiotidae Stec, Vecchi et Michalczyk, 2020. Soon after, Guidetti *et al*. (2021) described an additional new *Crenubiotus* species from Germany, presenting its phylogenetic position that was congruent with the results provided by Stec *et al*. (2020*b*). Moreover, Guidetti *et al.* (2021) changed the name of the family Richtersiidae into Richtersiusidae Guidetti, Schill, Giovannini, Massa, Goldoni, Ebel, Förschler, Rebecchi et Cesati, 2021, because the former was a junior homonym of a nematode family. As a result of the systematic and nomenclatural changes, the family Richtersiusidae now includes two genera, *Richtersius* and *Diaforobiotus*, with the first of these comprising only two nominal species, *R. coronifer* and *R. ziemowiti*.

In this study, we re-examined the *Richtersius* population from Greece previously studied by Stec *et al*. (2020*a*) and described and designated it as a new species. Our research utilised an integrative taxonomy approach, involving detailed morphological and morphometric examination under phase contrast light microscopy (PCM) as well as scanning electron microscopy (SEM), linked with genetic data already obtained for this population in the study mentioned above.

MATERIAL AND METHODS

Samples and specimens – A mixed moss and lichen sample was collected on 4 July 2015 from a tree in a forest on the Greek island of Crete (Omalos, Chania). The sample was examined for tardigrades using the protocol by Dastych (1980), with modifications described in detail in Stec *et al.* (2015) for the purpose of the earlier study by Stec *et al.* (2020*a*). Animals and eggs were extracted from the sample and split into several groups for specific analyses, i.e. morphological analysis in PCM and SEM, as well as DNA sequencing (for details, see "Material examined" provided below in the result section of the species description).

Microscopy and imaging – Specimens for light microscopy were mounted on microscope slides in a small drop of Hoyer's medium and secured with a coverslip, following Morek *et al*. (2016) protocol. Slides were examined under an Olympus BX53 light microscope with phase contrast associated with an Olympus DP74 digital camera. To obtain clean and extended specimens for SEM analysis, tardigrades were processed according to Stec *et al*. (2015) protocol. Specimens were examined under high vacuum in a Versa 3D DualBeam Scanning Electron Microscope at the ATOMIN facility of the Jagiellonian University, Kraków, Poland. All figures were assembled in Corel Photo-Paint X8. For structures that could not be focused in a single photograph, a stack of 2–10 images were taken with an equidistance of ca. 0.2 μm and assembled manually into a single deep-focus image.

Morphometrics and morphological nomenclature – All measurements are given in micrometres (μm). The sample size was adjusted following recommendations by Stec *et al.* (2016). Structures were measured only if their orientation was suitable. Body length was measured from the anterior to the posterior extremity of the body, excluding the hind legs. The terminology used to describe the oral cavity armature and eggshell morphology follow Kaczmarek and Michalczyk (2017), Guidetti *et al.* (2016) and Stec *et al.* (2020*a*, *b*). Macroplacoid length sequence is given according to Kaczmarek *et al.* (2014). Buccal tube length and the level of the stylet support insertion point were measured according to Pilato (1981). The *pt* index is the ratio of the length of a given structure to the length of the buccal tube expressed as a percentage (Pilato 1981). Buccal tube width was measured as the external and internal diameter at the level of the stylet support insertion point. Heights of claw branches were measured according to Kaczmarek and Michalczyk (2017), i.e. from the base of the claw (*i.e.* excluding the lunulae) to the top of the branch, including accessory points. The claw common tract index (*cct*) is the proportion of the height of the common tract of the claw (measured from the claw base to the separation point between the first and the second branch) to the total claw height expressed as a percentage (Guidetti *et al.* 2016). The description of cuticular bars on legs follows Kiosya *et al.* (2021). The distance between egg processes was measured as the shortest span between the base edges of the two closest processes. Following Stec *et al.* (2020*a*), we measured six additional traits: cuticular pore density (PD, the number of pores per 2500 μm^2 counted within a rectangle on the dorsal cuticle between legs III and IV), pore size (PS, measured as largest diameter; ten pores per measured specimen), the number of teeth on the external and internal lunulae III (ExtT and IntT, respectively) and the number of teeth on the anterior and posterior lunulae IV (AntT and PosT, respectively). Morphometric data were handled using the "Parachela" ver. 1.8 template available from the Tardigrada Register (Michalczyk & Kaczmarek 2013) and are provided as Supplementary Material (SM.01 and SM.02). Tardigrade taxonomy follows Bertolani *et al.* (2014), Stec *et al.* (2020b) and Guidetti *et al.* (2021).

Comparative molecular analysis – For molecular comparisons, all published sequences of the 18S rRNA, 28S rRNA, ITS-2 and COI markers of suitable length, and homological fragments for the genus *Richtersius* were downloaded from GenBank (Table 1). The sequences were aligned using the default settings (in the case of COI and ITS-2) and the Q-INS-I method (in the case of 18S rRNA, 28S rRNA) of *MAFFT* version 7 (Katoh *et al.* 2002, Katoh & Toh 2008) and manually checked against non-conservative alignments in *BioEdit*. The aligned sequences were trimmed to: 764 (18S rRNA), 762 (28S rRNA), 442 (ITS-2), 641 (COI), bp. All COI sequences were translated into protein sequences in *MEGA7* version 7.0 (Kumar *et al.* 2016) to check against pseudogenes. Uncorrected pairwise distances were calculated using *MEGA* and are provided as Supplementary Material (SM.03).

Table 1. GenBank accession numbers for sequences of species of the genus *Richtersius* analysed in this study. Bold numbers indicate sequences of the new species that were assigned as GR.008 population in Stec *et al.* (2020*a*) whereas italic numbers indicate type or neotype populations.

Species	18S rRNA	28S rRNA	ITS-2	COI	Source
R. coronifer s.s. (Richters, 1903)	*MH681760*	*MH681757*	*MH681763*	*MH676053*	Stec *et al.* (2020*a*)
R. aff. *coronifer* IT.120	MH681761	MH681758	MH681764	MH676054	Stec *et al.* (2020*a*)
R. aff. *coronifer* PL.246	MH681762	MH681759	MH681765	MH676055	Stec *et al.* (2020*a*)
R. aff. *coronifer* IT.317	MK211387	MK211385	MK211382–3	MK214326–8	Stec *et al.* (2020*a*)
R. tertius sp. n.	**MK211386**	**MK211384**	**MK211380–1**	**MK214323–5**	Stec *et al.* (2020*a*)
R. ziemowiti Kayastha *et al.*, 2020*a*	*MT241891*	*MT241892–5*	*MT241896*	*MT246504*	Kayastha *et al.* (2020a)
R. aff. *coronifer*	EU266930–1	–	–	–	Sands *et al.* 2008
R. aff. *coronifer*	AY582123	–	–	–	Jørgensen & Kristensen (2004)
R. aff. *coronifer*	HQ604987–8	–	–	–	Bertolani *et al.* 2014
R. cf. *coronifer*	KT778706–13	–	–	KT778683–94	Guidetti *et al.* 2016
R. aff. *coronifer*	–	–	–	EU244606–7	Schill and Jönsson (unpubl.)
R. cf. *coronifer*	–	–	–	EU251383–5	Faurby *et al.* (2008)
R. aff. *coronifer*	–	–	–	AY598780–1	Guidetti *et al.* (2005)
Richtersius sp.	–	–	–	JX865316	Czechowski *et al.* (2012)
Richtersius sp.	–	–	–	GU237485, GU339056*	Li and Xiao (unpubl.)

*mislabelled in GenBank as *Paramacrobiotus richtersi* (Murray, 1911) (see also Stec *et al.* 2020*a*)

RESULTS

Phylum: Tardigrada Doyère, 1840
Class: Eutardigrada Richters, 1926
Order: Parachela Schuster, Nelson, Grigarick et Christenberry, 1980
Superfamily: Macrobiotoidea Thulin, 1928
Family: Richtersiusidae Guidetti, Schill, Giovannini, Massa, Goldoni, Ebel, Förschler, Rebecchi et Cesari, 2021
Genus: *Richtersius* Pilato et Binda, 1989

Richtersius tertius sp. n.
(Figs 1–7)

Richtersius sp. 7 (GR.008; MK214323–5) in Stec *et al.* 2020*a*
Richtersius sp. (OTU6; MK214323–5) in Kayastha *et al.* 2020*a*

Etymology: The name "*tertius*" refers to the fact that the new species is the third formally described in the genus *Richtersius*.

Material examined: 171 animals and 73 eggs: specimens mounted on microscope slides in Hoyer's medium (134 adult animals + 12 hatchlings + 59 eggs), fixed on SEM stub (12 + 9 + 14), and used for DNA extraction and sequencing (4 + 0 + 0; in Stec *et al.* 2020*a*).

Description of the new species – Adults (measurements and statistics in Table 2).

Table 2. Measurements [in μm] and *pt* values of selected morphological structures of adults of *Richtersius tertius* sp. n. excluding hatchlings. (Specimens mounted in Hoyer's medium; N = number of specimen/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	608–904	–	753		75		866	
Buccal tube									
Buccal tube length	30	65.1–90.8	–	77.9	–	5.7	–	81.2	–
Stylet support insertion point	30	43.1–59.4	*65.3–68.9*	52.5	*67.3*	4.1	*1.1*	53.6	*66.0*
Buccal tube external width	30	4.0–7.3	*5.6–8.8*	5.9	*7.5*	0.8	*0.8*	6.1	*7.5*
Buccal tube internal width	30	1.6–2.3	*2.0–3.1*	1.9	*2.5*	0.2	*0.3*	1.9	*2.3*
Ventral lamina length	30	24.6–40.1	*37.8–48.9*	34.3	*43.9*	3.6	*2.8*	32.5	*40.0*
Placoid lengths									
Macroplacoid 1	30	10.3–15.2	*13.7–19.5*	12.7	*16.4*	1.1	*1.7*	14.9	*18.3*
Macroplacoid 2	30	5.3–9.9	*6.9–13.1*	8.5	*10.9*	0.8	*1.1*	8.6	*10.6*
Macroplacoid row	30	19.1–25.4	*25.3–32.6*	22.2	*28.6*	1.4	*2.0*	25.4	*31.3*
Claw I heights									
External base	24	9.1–15.8	*12.8–19.9*	12.1	*15.4*	1.6	*1.7*	12.2	*15.0*
External primary branch	24	16.1–25.0	*23.8–31.6*	21.5	*27.4*	1.9	*2.1*	23.1	*28.4*

Table 2 (continued)

Character	N	Range		Mean		SD		Holotype	
		µm	*pt*	µm	*pt*	µm	*pt*	µm	*pt*
External secondary branch	24	12.9–20.2	*19.0–25.8*	17.3	*22.0*	1.7	*1.8*	17.5	*21.6*
External base/primary branch (cct)	24	48.4–66.7	–	56.5	–	4.6	–	52.8	–
Internal base	27	7.4–14.6	*11.4–18.6*	11.4	*14.5*	1.9	*1.9*	10.6	*13.1*
Internal primary branch	27	15.4–24.8	*22.9–29.9*	20.7	*26.4*	2.2	*1.9*	22.6	*27.8*
Internal secondary branch	27	12.6–20.2	*17.2–25.1*	16.4	*21.0*	2.1	*2.0*	15.7	*19.3*
Internal base/primary branch (cct)	27	43.5–65.8	–	54.9	–	5.6	–	46.9	–
Claw II heights									
External base	28	8.6–16.2	*13.0–20.3*	12.7	*16.2*	1.8	*1.7*	15.0	*18.5*
External primary branch	28	17.1–26.6	*23.8–33.7*	22.2	*28.4*	2.1	*2.5*	26.6	*32.8*
External secondary branch	28	13.5–21.6	*18.0–27.8*	18.2	*23.3*	1.7	*2.0*	20.2	*24.9*
External base/primary branch (cct)	28	47.9–74.2	–	57.2	–	5.5	–	56.4	–
Internal base	28	8.3–15.8	*12.5–19.9*	11.7	*15.0*	1.8	*1.7*	13.2	*16.3*
Internal primary branch	28	15.4–25.3	*23.7–31.9*	21.2	*27.3*	2.3	*2.1*	25.3	*31.2*
Internal secondary branch	28	11.7–20.5	*18.0–26.0*	16.9	*21.7*	2.0	*2.0*	20.5	*25.2*
Internal base/primary branch (cct)	28	46.4–65.0	–	55.0	–	5.3	–	52.2	–
Claw III heights									
External base	26	9.3–15.2	*13.5–18.8*	12.6	*16.3*	1.5	*1.6*	14.0	*17.2*
External primary branch	26	16.9–26.3	*24.1–32.4*	22.3	*28.8*	2.3	*2.4*	26.3	*32.4*
External secondary branch	26	12.1–21.1	*18.0–26.1*	17.9	*23.1*	1.9	*2.0*	21.1	*26.0*
External base/primary branch (cct)	26	51.7–66.4	–	56.5	–	3.7	–	53.2	–
Internal base	24	9.0–15.0	*12.3–18.4*	11.8	*15.1*	1.6	*1.7*	13.3	*16.4*
Internal primary branch	24	16.2–25.3	*23.3–32.6*	21.7	*27.8*	2.3	*2.4*	25.3	*31.2*
Internal secondary branch	24	12.0–20.8	*17.9–27.1*	17.2	*22.1*	2.2	*2.5*	20.8	*25.6*
Internal base/primary branch (cct)	24	47.1–64.4	–	54.3	–	4.5	–	52.6	–
Claw IV heights									
Anterior base	23	10.6–18.3	*15.7–23.1*	14.4	*18.6*	1.8	*1.8*	15.3	*18.8*
Anterior primary branch	23	21.5–31.8	*29.4–41.5*	27.4	*35.4*	2.8	*2.9*	29.0	*35.7*
Anterior secondary branch	23	14.4–23.4	*19.8–31.5*	19.8	*25.5*	2.9	*2.9*	22.7	*28.0*
Anterior base/primary branch (cct)	23	46.5–57.5	–	52.5	–	2.9	–	52.8	–
Posterior base	24	9.2–18.4	*14.1–23.2*	13.8	*17.6*	2.3	*2.3*	14.3	*17.6*
Posterior primary branch	24	19.7–30.4	*28.7–39.0*	26.7	*34.3*	2.9	*2.6*	28.1	*34.6*
Posterior secondary branch	24	13.9–24.1	*20.9–29.2*	19.3	*24.8*	2.6	*2.6*	22.1	*27.2*
Posterior base/primary branch (cct)	24	43.6–63.9	–	51.4	–	5.2	–	50.9	–
Number of teeth on external lunula III	29	7–19	–	12	–	3	–	10	–
Number of teeth on internal lunula III	28	7–16	–	11	–	2	–	10	–
Number of teeth on anterior lunula IV	28	9–23	–	14	–	3	–	13	–
Number of teeth on posterior lunula IV	28	10–22	–	14	–	3	–	17	–

Body yellow, after fixation in Hoyer's medium, all specimens become transparent (Fig. 1A). Eyes present both in live animals and specimens mounted in Hoyer's medium. Body and leg cuticle without granulation (Figs 1A, 3A–D).

Claws slender, primary branches with distinct accessory points (Fig. 3A–D) and a system of internal septa as described by Lısı *et al.* (2020). Secondary branches approximately 80% of the length of the primary branches. An evident stalk system connecting the claws to the lunulae is visible under PCM and SEM (Fig. 3A–D). The stalk system consists of a thin laminar stalk connecting the claw to the lunula and two posterior lateral extensions (Fig. 3A–D). The distal tips of these lateral extension under PCM appear to be connected to the stalk where it contacts the lunula (Fig. 3A–B) whereas under SEM the stalk system is visible as a cuticular plate with a protuberant laminar stalk (Fig. 3C–D). Lunulae large, smoothly unified with the leg cuticle and with a crown of long, numerous and densely arranged spikes (2.0–3.1 μm long) (Fig. 3A–D). Lunulae I–III trapezoidal, whereas lunulae IV ovate (Fig. 2A–F). Divided cuticular bars, double muscle attachments, and horseshoe structures are visible in PCM (Fig. 3A–B).

Mouth antero-ventral. Sensory lobes merged into a single circular sensory field surrounding the mouth (Fig. 4A). Anteriorly, the mouth begins with fused peribuccal lamellae forming a circular velum/lamina which is posteriorly folded into a pre-mouth ventricle (Fig. 4A–B). With the exception of the second band of teeth, which is rarely and only faintly visible in larger specimens, the oral cavity armature is not visible under PCM. (Fig. 5B–C). Under SEM, the oral cavity armature is clearly seen to be composed of three bands of teeth (Fig. 4A–B). The first and the second band form continuous rings around the axis of the

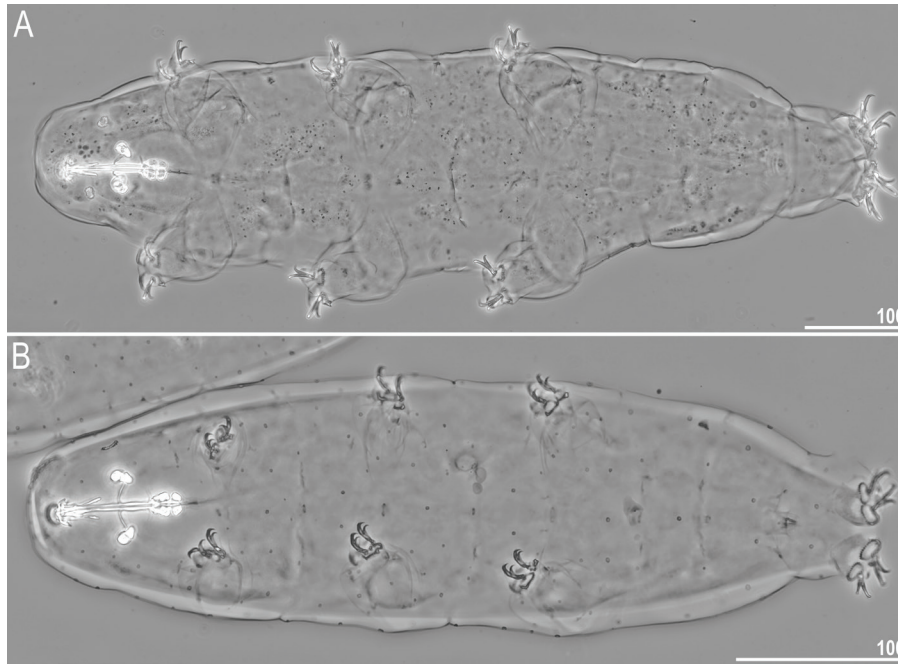

Fig. 1. *Richtersius tertius* sp. n. habitus. A = adult, dorso-ventral projection (holotype, PCM); B = hatchling, dorso-ventral projection (PCM). Scale bars in μm

mouth, whereas the third band is divided into a dorsal and a ventral portion (Fig. 4A–B). The first band of teeth lies on the inner surface of the velum and is composed of numerous small granular cones forming about twenty irregular rows with slightly bigger teeth posi-

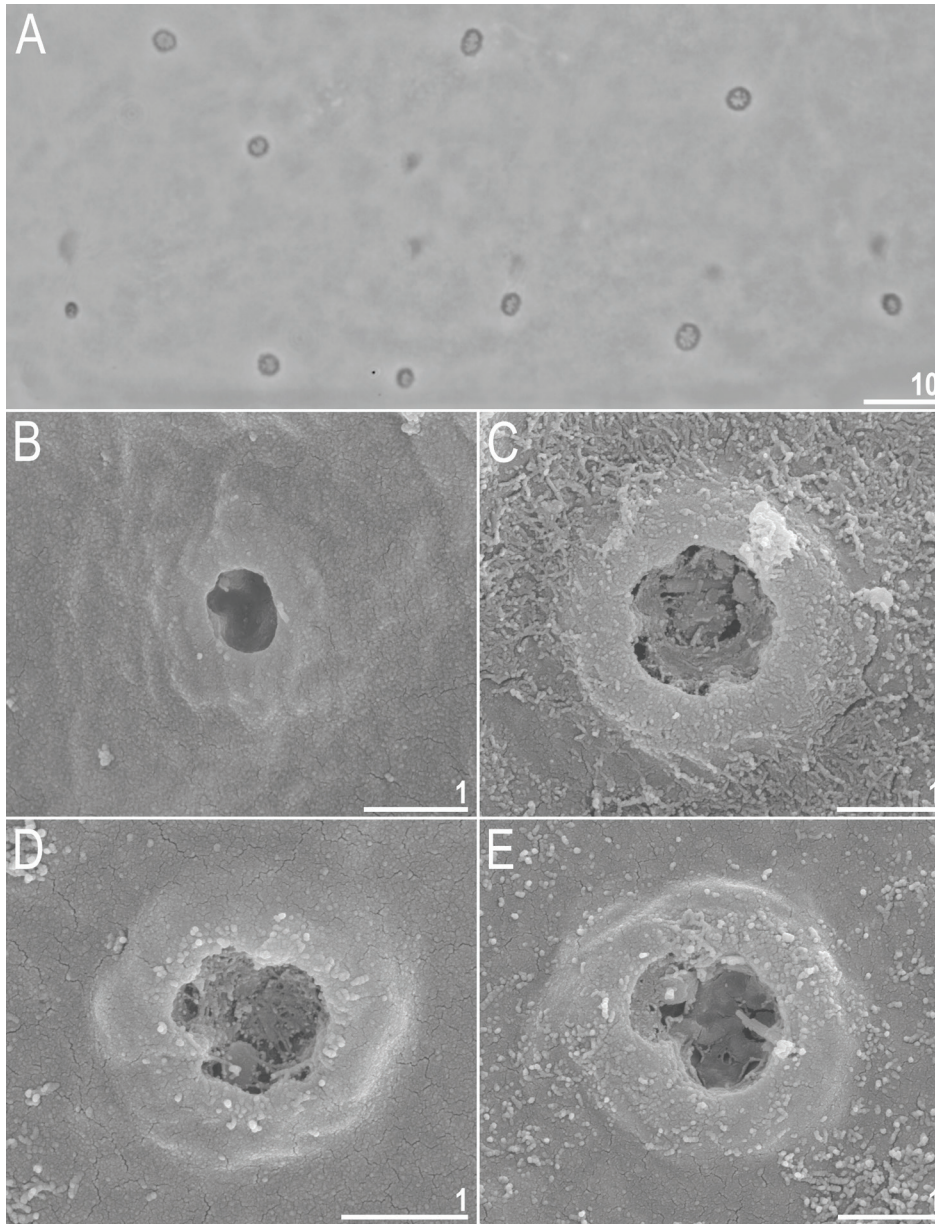

Fig. 2. *Richtersius tertius* sp. n. pores. A = pores on the hatchling dorsal cuticle (PCM); B–E = pores on the hatchling dorsal cuticle (SEM). Scale bars in μm

tioned on the edge closest to the velum (Fig. 4A–B). The second band of teeth consists of about ten irregular rows of densely packed, elongate, sharp cones which lie on a cuticular fold protruding from the pre-mouth ventricle (Fig. 4A–B). The discontinuous third band of teeth is situated between the second band of teeth and buccal tube opening and is divided into a dorsal and a ventral portion, both in the form of a single large tooth, resembling a beak (Fig. 4A–B). The ventral tooth resembles an isosceles trapezium standing on its longer base, with a ragged upper edge. The dorsal tooth is semicircular in shape, with a crescent-shaped indentation in its middle (Fig. 4 A–B).

Table 3. Measurements [in μm] and *pt* values of selected morphological structures of hatchlings of *Richtersius tertius* sp. n. (Specimens mounted in Hoyer's medium; N = number of specimen/structures measured, Range refers to the smallest and the largest structure among all measured specimens; SD = standard deviation).

Character	N	Range		Mean		SD	
		μm	*pt*	μm	*pt*	μm	*pt*
Body length	6	433–505	*738–922*	474	*834*	27	*81*
Buccal tube							
Buccal tube length	6	51.4–67.8	–	57.3	–	7.6	–
Stylet support insertion point	5	34.4–46.3	*66.8–68.3*	39.1	*67.7*	6.1	*0.7*
Buccal tube external width	6	3.6–5.6	*6.5–10.9*	4.6	*8.1*	0.7	*1.6*
Buccal tube internal width	6	1.5–1.8	*2.7–3.1*	1.7	*2.9*	0.1	*0.2*
Ventral lamina length	5	21.5–32.1	*41.8–47.3*	26.2	*45.2*	5.0	*2.3*
Placoid lengths							
Macroplacoid 1	6	11.0–12.4	*18.3–22.3*	11.6	*20.4*	0.6	*1.8*
Macroplacoid 2	6	5.1–6.9	*9.9–13.2*	6.5	*11.4*	0.7	*1.6*
Macroplacoid row	6	17.3–19.5	*28.6–35.8*	18.6	*32.7*	0.8	*3.0*
Claw I heights							
External base	4	7.6–9.1	*13.8–17.7*	8.5	*16.2*	0.6	*1.7*
External primary branch	4	14.2–15.5	*27.2–29.9*	14.9	*28.4*	0.5	*1.2*
External secondary branch	4	10.7–11.8	*20.6–22.1*	11.2	*21.3*	0.5	*0.6*
External base/primary branch (cct)	4	50.7–64.1	–	57.3	–	5.6	–
Internal base	5	7.2–8.7	*11.0–15.2*	7.8	*13.7*	0.6	*1.8*
Internal primary branch	5	12.9–16.8	*22.4–28.7*	14.6	*25.5*	1.5	*2.3*
Internal secondary branch	5	9.1–12.8	*16.9–22.7*	11.2	*19.6*	1.3	*2.5*
Internal base/primary branch (cct)	5	49.3–57.4	–	53.5	–	3.2	–
Claw II heights							
External base	6	8.0–9.3	*12.1–16.9*	8.6	*15.2*	0.5	*1.9*
External primary branch	6	14.1–16.6	*21.3–32.0*	15.2	*26.9*	1.0	*3.8*
External secondary branch	6	11.0–12.9	*16.6–24.7*	11.8	*20.9*	0.9	*2.8*
External base/primary branch (cct)	6	52.4–60.4	–	56.7	–	3.0	–

Table 3 (continued)

Character	N	Range		Mean		SD	
		μm	*pt*	μm	*pt*	μm	*pt*
Internal base	6	7.5–8.3	*11.1–16.0*	7.8	*13.7*	0.3	*1.9*
Internal primary branch	6	13.2–14.4	*20.4–27.8*	14.0	*24.7*	0.5	*3.1*
Internal secondary branch	6	9.7–12.1	*15.3–23.3*	11.1	*19.6*	1.0	*2.9*
Internal base/primary branch (cct)	6	52.1–62.9	–	55.7	–	4.2	–
Claw III heights							
External base	5	8.0–9.7	*14.0–17.6*	9.0	*16.3*	0.7	*1.5*
External primary branch	5	14.6–16.9	*24.9–31.6*	15.6	*28.3*	1.0	*2.4*
External secondary branch	5	9.8–13.7	*19.1–23.1*	11.5	*20.8*	1.5	*1.5*
External base/primary branch (cct)	5	54.8–63.8	–	57.8	–	3.8	–
Internal base	5	6.8–8.6	*12.7–16.4*	8.0	*14.5*	0.8	*1.5*
Internal primary branch	5	13.8–16.3	*24.0–28.2*	14.7	*26.6*	0.9	*1.6*
Internal secondary branch	5	9.4–13.6	*18.3–21.0*	10.9	*19.7*	1.7	*1.2*
Internal base/primary branch (cct)	5	49.3–59.0	–	54.4	–	4.0	–
Claw IV heights							
Anterior base	3	8.5–11.5	*16.3–17.0*	9.7	*16.6*	1.6	*0.3*
Anterior primary branch	3	18.4–21.0	*31.0–37.1*	19.5	*33.8*	1.3	*3.1*
Anterior secondary branch	3	13.0–15.6	*23.0–25.4*	13.9	*24.0*	1.5	*1.3*
Anterior base/primary branch (cct)	3	44.5–54.8	–	49.4	–	5.1	–
Posterior base	5	7.7–10.8	*15.0–15.9*	8.6	*15.4*	1.3	*0.4*
Posterior primary branch	5	16.3–22.6	*31.7–34.1*	18.5	*33.3*	2.4	*0.9*
Posterior secondary branch	5	11.6–16.9	*22.6–28.5*	13.6	*24.5*	2.2	*2.4*
Posterior base/primary branch (cct)	5	44.5–47.9	–	46.3	–	1.5	–
Number of teeth on external lunula III	6	10–15	–	12	–	2	–
Number of teeth on internal lunula III	6	7–15	–	13	–	3	–
Number of teeth on anterior lunula IV	5	11–16	–	13	–	2	–
Number of teeth on posterior lunula IV	6	11–18	–	14	–	2	–
Pore density	6	3–6	–	5	–	1	–
Pore size	6	1.2–4.1	–	2.8	–	0.6	–

Buccal apparatus massive. The oral cavity is followed by a system of massive apophyses forming a buccal crown (Fig. 5A–C). Anteriorly, the system consists of dorso-lateral and ventro-lateral triangular apophyses (Fig. 5A, C). The dorsal and ventral apophyses, are composed of anteriorly positioned large cuticular hooks followed by longitudinal crests which in case of ventral apophyses might be homologous to the ventral lamina found in ordinary macrobiotids (Fig. 5B). The hook in the ventral apophyses is smaller than the dorsal

hook (Fig. 5B). The buccal tube wall exhibits variable thickness, but the internal diameter of the buccal tube is almost uniformly narrow (Fig. 5A). From mouth opening to the stylet support insertion point, the thickens of the buccal tube wall increases only slightly before also slightly expanding to its greatest thickness beyond this point before then shrink posteriorly (Fig. 5A). Pharynx spherical, with bilobed apophyses, three anterior cuticular spikes (typically only two are visible in any given plane) and two granular macroplacoids (2<1). The first and the second macroplacoid with a faint constriction positioned centrally and subterminally, respectively (Fig. 5A–E).

Hatchlings (measurements and statistics in Table 3). Same as adults, except for smaller body size (Fig. 1B) and round pores (1.2–4.1 μm in diameter) with wavy edges, clearly visible both under PCM and SEM, scattered randomly on the entire body cuticle (Figs 1B, 2A–E), with a PD range of 3–6.

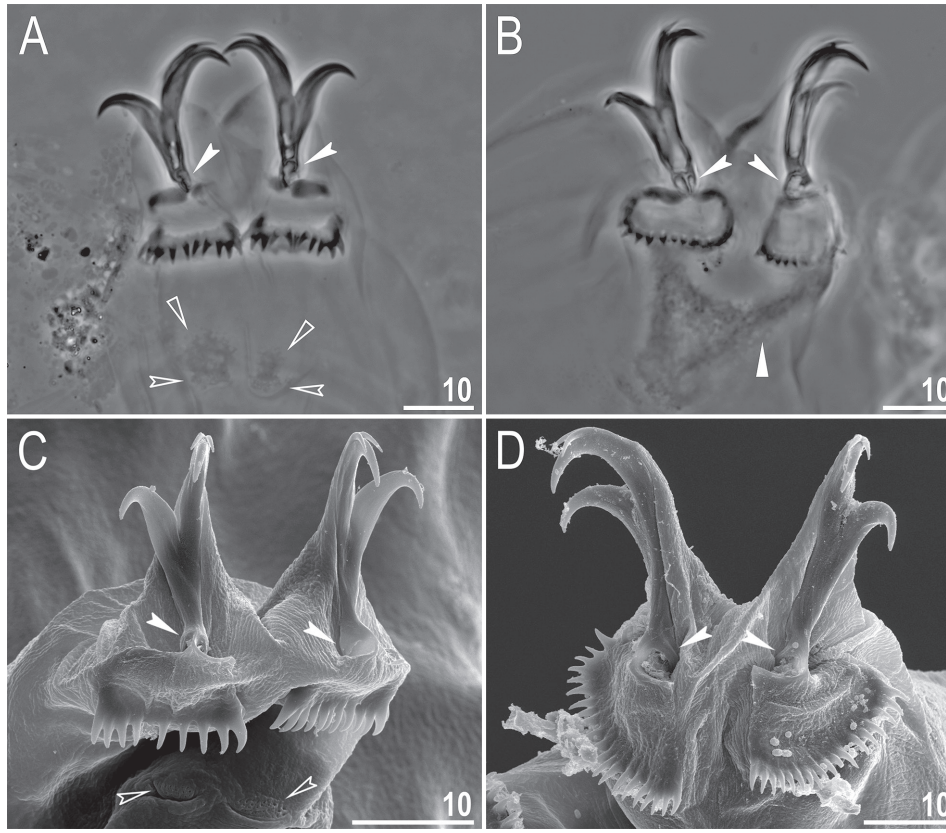

Fig. 3. *Richtersius tertius* sp. n. claws. A–B = claws II and IV (PCM); C–D = claws III and IV (SEM). Filled indented arrowheads indicate lateral expansions positioned posteriorly to the laminar stalk connecting the claw to the lunula, empty indented arrowheads indicate the double muscle attachments under the claws, empty flat arrowheads indicate the divided cuticular bar, filled flat arrowhead indicates the horseshoe structure connecting the anterior and posterior claws. Scale bars in μm

Table 4. Measurements [in μm] of the eggs of *Richtersius tertius* sp. n. (eggs mounted in Hoyer's medium; process base/height ratio is expressed as percentage; 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	30	117.4–155.3	139.2	7.9
Egg full diameter	30	149.8–188.2	165.9	10.1
Process height	90	13.7–27.4	20.0	2.9
Process base width	90	3.0–6.5	4.7	0.9
Process base/height ratio (%)	90	14–42	24	5
Inter-process distance	90	5.2–13.1	8.3	1.7
Number of processes on the egg circumference	30	32–44	38.0	3.5

Eggs (measurements and statistics in Table 4). Large, oval, light yellow, laid freely (Figs 6A–B, 7A). The surface between processes is smooth but with refracting dots faintly visible only under PCM, but difficult to observe because of the amount of debris that is typically attached to the egg surface (Figs 6C, 7A–D). Processes in the shape of elongated, thin, cones with a ragged surface (Figs 6 D–E, 7B–D). Terminal portions of the egg processes are flexible, sometimes divided into two or three short filaments (Figs 6D–E, 7A–D). Terminal discs or spatulas absent.

Reproduction – The new species is dioecious: both males with testes and females with ovaries were recorded within the examined population (Stec *et al.* 2020*a*).

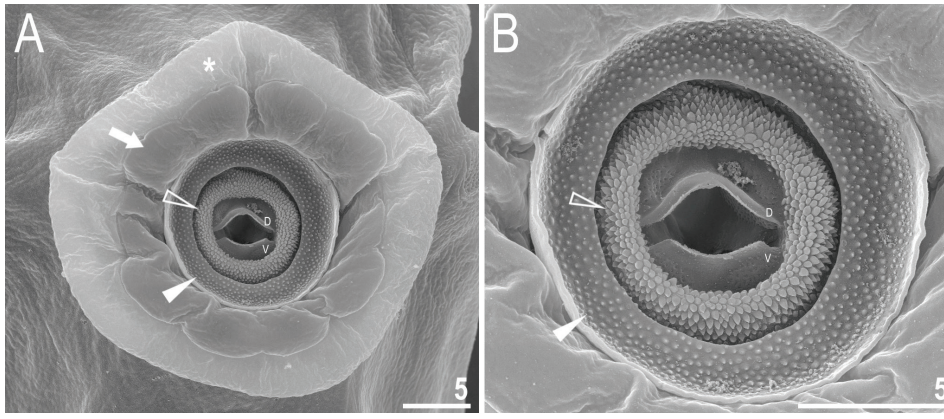

Fig. 4. *Richtersius tertius* sp. n. mouth opening with peribuccal structures and oral cavity armature of an adult (SEM). A = general view of the mouth opening; B = details of the oral cavity armature. Asterisk indicates the circular sensory field, filled arrow indicates the divided peribuccal velum/lamina, filled flat arrowheads indicate the first band of teeth, empty flat arrowheads indicate the teeth of the second band, dorsal and ventral teeth of third band are marked with D and V, respectively. Scale bars in μm

DNA sequences – The DNA sequences of four molecular markers (18S rRNA, 28S rRNA, ITS-2 and COI) associated with the species description have been previously published by Stec *et al.* (2020*a*). The respective GenBank accession numbers are given in Table 1.

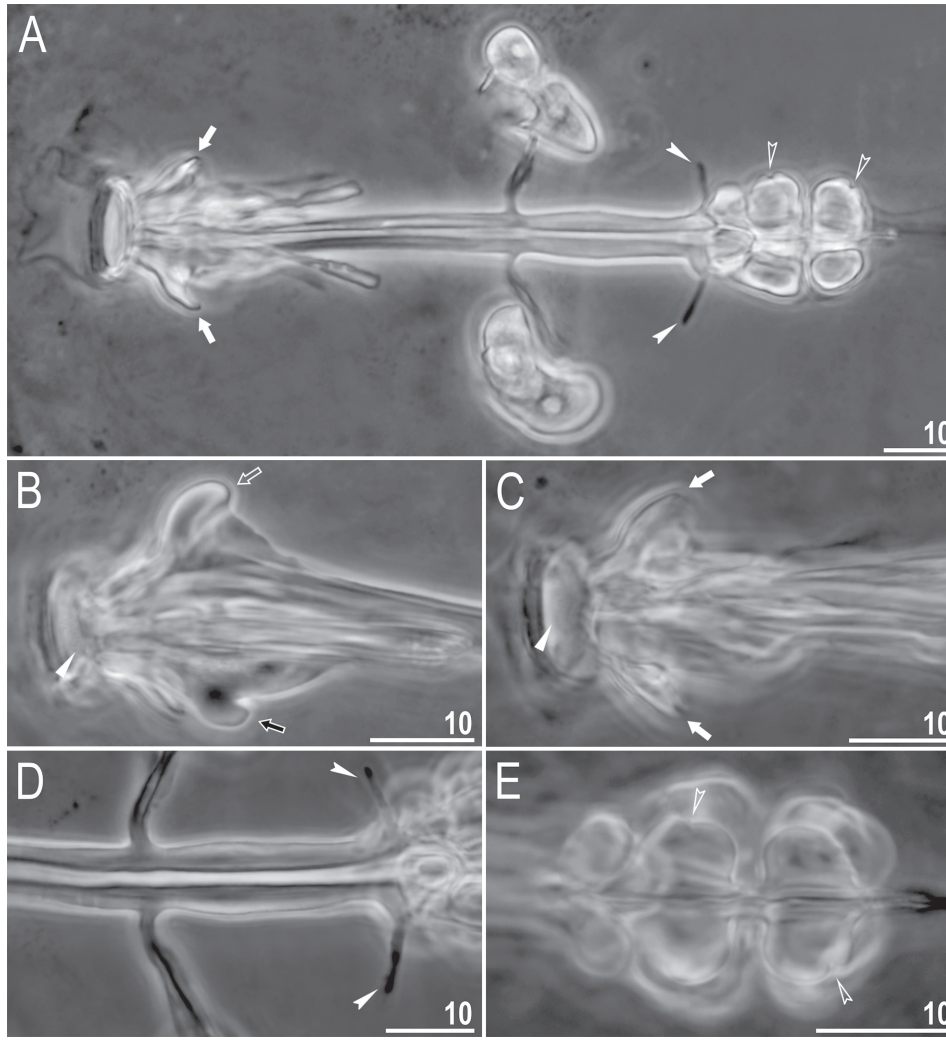

Fig. 5. *Richtersius tertius* sp. n. buccal apparatus (PCM). A = dorsal projection of the entire buccal apparatus; B = later view of the buccal crown; C = ventral view of the buccal crown; D = stylet support insertion point of the buccal apparatus; E = macroplacoid morphology visible in ventral view. Filled arrows indicate the ventro-lateral triangular apophysis, filled indented arrowheads indicate dorsal spikes, empty indented arrowheads indicate constrictions in the macroplacoids, the empty arrow indicates the cuticular hook on the dorsal apophysis, the black filled arrow indicates the cuticular hook on the ventral apophysis, filled flat arrowheads indicate the second band of teeth in the oral cavity. Scale bars in μm

Locality: 35°16′16′′N, 23°57′41′′E; Greece, Crete, Chania, Omalos; forest; moss and lichen from a tree in a forest; coll. 04.07.2015 by Małgorzata Mitan and Małgorzata Osielczak.

Type depositories: The holotype (slide GR.008.15 with 4 paratypes), as well as 162 paratypes (slides: GR.008.*, where the asterisk can be substituted by any of the following numbers, 03–29; SEM stub: 19.12) and 73 eggs (slides: GR.008.* 30–33; SEM stub: 19.12) are deposited at the Institute of Zoology and Biomedical Research, Jagiellonian University, Gronostajowa 9, 30–387, Kraków, Poland whereas 51 paratypes (slides: GR.008.*, where the asterisk can be substituted by any of the following numbers, 20–29) and 31 eggs (slides: GR.008.* 34–37) are deposited at the Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Sławkowska 17, 31-016, Kraków, Poland.

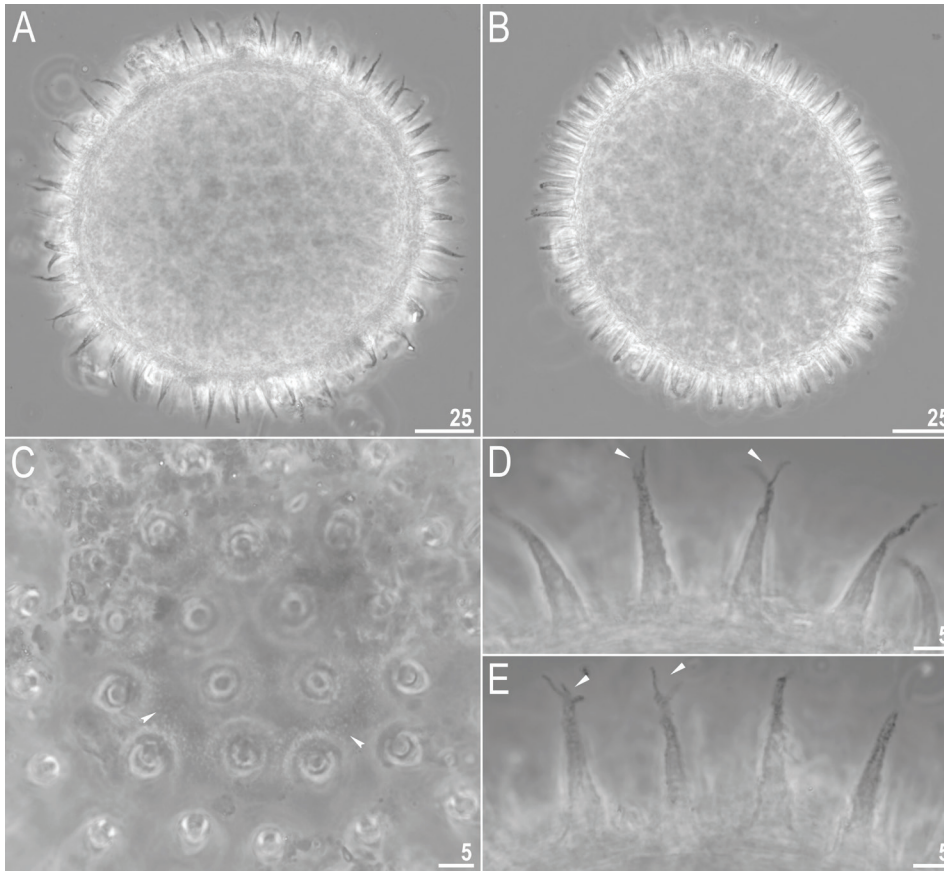

Fig. 6. *Richtersius tertius* sp. n. egg chorion morphology (PCM). A–B = egg midsection under 400× magnification; C = egg surface under 1000× magnification; D–E = egg processes midsection under 1000× magnification. Filled flat arrowheads indicate flexible terminal portion of the egg process, filled indented arrowheads indicate the refracting dots covering the egg surface between processes (in the majority of eggs this character is not visible as the egg surface is often covered by dirt). Scale bars in μm

DISCUSSION

Phenotypic differential diagnosis

As mentioned above, the genus *Richtersius*, currently comprises only two species, *R. coronifer*, known only from Norway and Greenland (Stec *et al.* 2020*a*), and *R. ziemowiti*, known only from Nepal (Kayastha *et al.* 2020*a,b*). The new species differs from:

Richtersius coronifer by: the shape and size of cuticular pores (round pores, 1.2–4.1 μm in diameter, with wavy edges in *R. tertius* sp. n. *vs* round and oval pores, 0.35–0.69 μm in diameter, with smooth edges in *R. coronifer*), a lower PD (3–6 in the new species *vs* 60–88 in *R. coronifer*), a weakly developed thickness of the buccal tube wall posterior to the stylet support insertion point (the thickness well developed and obvious in *R. coronifer*), a more anteriorly positioned

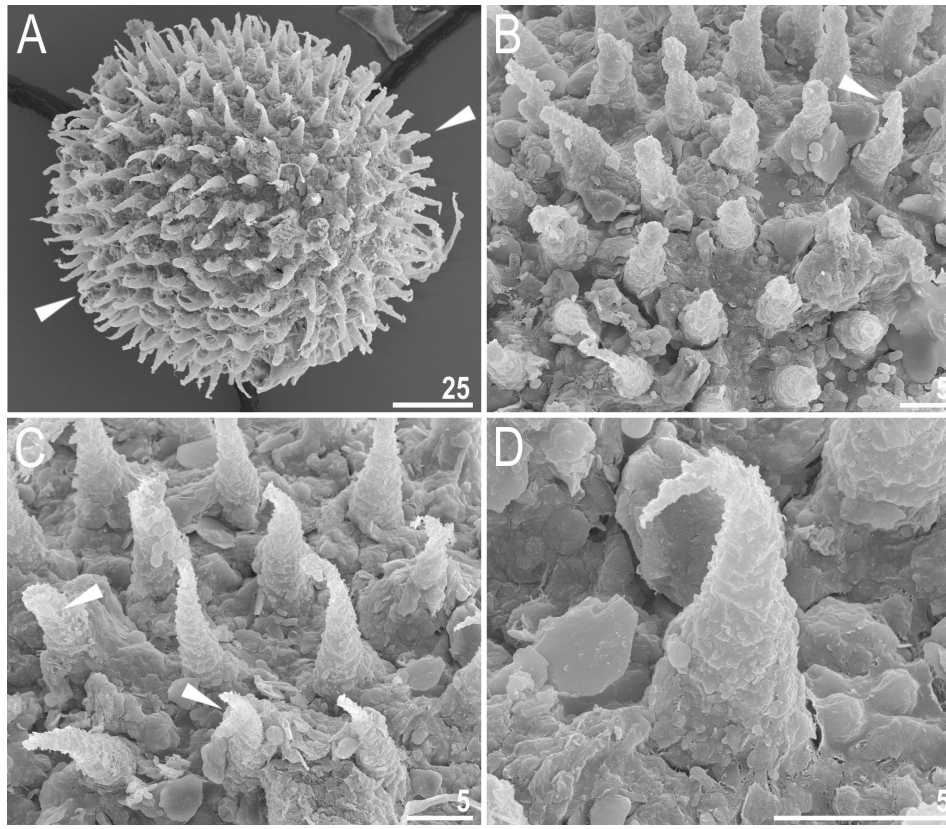

Fig. 7. *Richtersius tertius* sp. n. egg chorion morphology (SEM). A – entire egg; B–C = Details of egg surface and processes; D = magnification of the egg process. Filled flat arrowheads indicate the flexible terminal portion of the egg process. Scale bars in μm

stylet support insertion point (*pt = 65.3–68.9* in the new species *vs pt = 72.1–75.6* in *R. coronifer*), the morphology of the egg surface seen in PCM (with refracting dots in the new species *vs* smooth without refracting dots in *R. coronifer*), a smaller number of processes on the egg circumference (32–44 in *R. tertius* sp. n. *vs* 60–77 in *R. coronifer*), a smaller egg bare diameter (117.4–155.3 μm in the new species *vs* 173.2–233.4 μm in *R. coronifer*), and a smaller egg full diameter (149.8–188.2 μm in the new species *vs* 201.5–263.7 μm in *R. coronifer*).

Richtersius ziemowiti by: the shape of cuticular pores (round pores with wavy edges in *R. tertius* sp. n. *vs* round and oval pores with smooth margins in *R. ziemowiti*), a lower PD (3–6 in the new species *vs* 20–24 in *R. ziemowiti*), the morphology of the egg surface seen in PCM (with refracting dots in the new species *vs* without refracting dots in *R. ziemowiti*), a more anteriorly positioned stylet support insertion point (*pt = 65.3–68.9* in the new species *vs pt = 73.3–80.7* in *R. ziemowiti*), and a slightly lower *pt* value for external primary branches of claws I (*pt = 23.8–31.6* in the news species *vs pt = 33.2–42.9* in *R. ziemowiti*).

Genetic comparison

The ranges of uncorrected genetic p-distances between *Richtersius tertius* sp. n. and genotyped populations/species of the genus *Richtersius* are as follows:

- 18S rRNA: 0.4–2.2% (0.9% on average), with the most similar being *R.* aff. *coronifer* from Italy and Poland (MH681762, MK211387), *R.* aff. *coronifer* (HQ604987–8, KT778706–7) from Italy, *R.* cf. *coronifer* (KT778711) from Italy and the least similar being *R.* aff. *coronifer* (EU266931) from Greenland;
- 28S rRNA: 0.3–2.3% (1.6% on average), with the most similar being *R.* aff. *coronifer* from Italy (MK211385) and the least similar being *R.* aff. *coronifer* from Norway and *R. ziemowiti* (MH681757, MT241893) from Nepal;
- ITS-2: 7.7–35.6% (17.7% on average), with the most similar being *R.* aff. *coronifer* from Italy (MK211382, MK211383) and the least similar being *R. coronifer s.s.* from Norway (MH681763);
- COI: 16.8–25.3% (21% on average), with the most similar being *R.* aff. *coronifer* from Italy (MK214327) and the least similar being *Richtersius sp.* (GU339056) from China.

CONCLUSIONS

Thanks to morphological and morphometric analysis, as well as integration of these data with the DNA sequences published previously, we described a new *Richtersius* species that constitute the third formally named species in the genus. According to Stec *et al.* (2020*a*) and Kayastha *et al.* (2020*a*) there are at

least six additional putative new *Richtersius* species awaiting description. We believe that the implementation of integrative taxonomic tools and the collection of extensive sets of morphological and morphometric data, linked to molecular markers, will allow delimitations of further species within the genus.

*

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Supplementary materials

SM.01. Raw morphometric data for adults and eggs of *Richtersius tertius* sp. n.
SM.02. Raw morphometric data for hatchlings of *Richtersius tertius* sp. n.
SM.03. Uncorrected pairwise distances.