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A new sexually dimorphic *Diploechiniscus* species (Tardigrada: Echiniscidae) from Calvert Island (British Columbia, Canada)

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Abstract

An important, but generally overlooked source in intraspecific variability of characters used in tardigrades taxonomy is sexual dimorphism. Dioecious tardigrades species with unisexual or bisexual populations are common, but external sexual dimorphism is rarely observed. The discovery and integrative analysis of a *Diploechiniscus* species population with high sexual dimorphism from Calvert Island (Canada) has allowed its description as a new species. *Diploechiniscus dimorphus* n. sp. is characterized by a different chaetotaxy in males and females, and by enlarged cephalic sensory appendages in the males. The discovery of this new species shows that it is important to consider sexual dimorphism as a source of intraspecific variability in tardigrade taxonomy due to its implications on the use of traits commonly used to delineate species such as chaetotaxy. Lastly, the finding of individuals of *Diploechiniscus horningi*, a species previously synonymized with *Diploechiniscus oihonnae*, allows us to prove that it is genetically a separate species and to reinstate it.

Keywords

Echiniscidae, Tardigrada, sexual dimorphism, British Columbia, integrative taxonomy

Introduction

Tardigrades are a phylum of microinvertebrates known for their ability to resist desiccation. However, our knowledge of tardigrade alpha diversity remains incomplete and new species are discovered every year, in particular due to advances in our understanding of their intra- and-interspecific variability. Tardigrades are found all over the world in marine and limno-terrestrial habitats (Schill 2018). Currently, there are around 1400 officially recognized

tardigrade species (Guidetti and Bertolani 2005; Degma and Guidetti 2007, 2023), and this number increases annually. In particular, the number of tardigrade species reported using integrative taxonomy has also increased in recent years. The collection of data from such integrative investigations allowed for a more in-depth examination of phylogenetic relationships and species delimitations, and its utility has been repeatedly demonstrated (e.g., Surmacz et al. 2019; Bochnak et al. 2020; Kayastha et al. 2020; Tumanov 2020; Guidetti et al. 2021; Vecchi et al. 2023a).

The history of tardigrade taxonomy in Canada is summarized in Vecchi et al. (2022*a*,*b*) and Massa et al. (2024). This first Canadian surveys were published in the early twentieth-century. Richters (1908), identified three species collected from Vancouver, British Columbia during a Pacific expedition from 1896 to 1897 and Murray (1910), collected 31 species in Canada, although opportunities for collecting were limited due to a hurried journey across that part of the North American continent. To date, 60 species are known from British Columbia (Kaczmarek et al. 2016; Kayastha et al. 2021; Vecchi et al. 2022*a*,*b*), of which three Echiniscidae (Heterotardigrada) have their type locality in the province: *Echiniscus canadensis* Murray, 1910, *Echiniscus sylvanus* Murray, 1910, and *Echiniscus reymondi* Marcus, 1928 (originally identified by Murray 1910 as "*Echiniscus*, sp. ? (Plate XX. Fig. 45)" and described by Marcus). Other echiniscids have been found: Murray (1910) identified *Hypechiniscus gladiator* (Murra 1905), *Bryochoerus intermedius* (Murray 1910), *Diploechiniscus oihonnae* (Richters 1903), *Echiniscus bisetosus* (now synonymized with *Echiniscus blumi* Richters 1903), and finally two undescribed *Echiniscus* species ("*Echiniscus*, sp. ? (Plate XX. Fig. 44)" and ("*Echiniscus*, sp. ? (Plate XX. Fig. 46)"). Years later, Kathman and Dastych (1990) and Kathman and Cross (1991) performed a faunistic survey of tardigrades in Vancouver Island and recorded *Diploechiniscus horningi* (Schuster and Grigarick 1971), *Claxtonia mauccii* (Ramazzotti 1956), *Claxtonia wendti* (Richters 1903), *Acanthechiniscus goedeni* (Grigarick, Mihelčič and Schuster 1964), *Echiniscus quadrispinosus* Richters 1902, *Pseudechiniscus suillus* (Ehrenberg 1853), and *Testechiniscus laterculus* (Schuster, Grigarick and Toftner 1980). Most recently, Vecchi et al. (2022*b*) and Vecchi et al. (2023*b*) reported *Acanthechiniscus goedeni*, *Diploechiniscus* sp., and *Hypechiniscus gladiator*.

Diploechiniscus Vicente, Fontoura, Cesari, Rebecchi, Guidetti, Serrano and Bertolani 2013 is a genus of armoured tardigrades (Echiniscidae) erected for the species *Echiniscus oihonnae* Richters 1903 by Vicente et al. (2013) based on both morphological (dark eyes, double sculptured cuticle, median plates m1 and m2 subdivided) and molecular characters. In the same paper, *Echiniscus multispinosus* Da Cunha 1944 was designated as a junior synonym of *D. oihonnae*. Up to date *Diploechiniscus* contains three species *D. oihonnae, D. polygonalis* (Ito 1993) and *D. laterosetosus* (Ito 1993), recently added to the genus (Gąsiorek 2023). The genus was also briefly populated by another species, *D. horningi,* after Gąsiorek et al. (2019) moved it from *Echiniscus* to *Diploechiniscus*. This situation did not last long, as Kaczmarek et al. (2021) synonymized *D. horningi* with *D. oihonnae* and established a neotype for the latter species. Both Vicente et al. (2013) and Kaczmarek et al. (2021) pointed out the variability of chaetotaxy (i.e. the arrangement and shape of the body thin appendages, e.g. spines, filaments) of *D. oihonnae*, both in the presence/absence of a spine in B^d (the character originally used to differentiate *E. oihonnae* from *E. multispinosus*), not linked to any developmental stage or sex

(Kaczmarek et al. 2021), and in the presence of anormal individuals (e.g., without any dorsolateral filaments; Kaczmarek et al. 2021).

During a tardigrade survey of Calvert Island (British Columbia, Canada), we found individuals of two new species genetically clearly affiliated with *Diploechiniscus*, but morphologically well differentiated from all other species in the genus. One of the new species is also remarkable in showing sex-specific differences in chaetotaxy. We also found individuals from Vancouver Island morphologically similar to *D. oihonnae*, but belonging to a different species according to COI sequences.

In this paper, we present an integrative description of a new sexually dimorphic *Diploechiniscus* species from Calvert Island (British Columbia) based on DNA sequencing and morphological examination, and we reinstate *D. horningi* based on individuals from Vancouver Island (British Columbia).

Material and Methods

Samples and specimens

Tardigrade specimens were recovered from moss samples growing on a vertical rock outcrop near the Hakai Institute Calvert Island Field Station in 2021 and 2022 (Table 1). The Hakai field Station is located within the Hakai Lúxvbálís Conservancy of the Province of British Columbia on the central coast of British Columbia. The central coast of British Columbia extends from approximately the north end of Aristazabal Island at ∼52°49′ N to the entrance to Queen Charlotte Strait at ∼50°59′ N, excluding the offshore Haida Gwaii archipelago (Lindstrom et al. 2021). Additional moss samples were obtained during a survey of the Mount Arrowsmith region, Vancouver Island by the Royal BC Museum Department of Botany.

Samples were examined for tardigrades using the protocol by Degma (2018) and the animals were divided into three groups for specific analyses, i.e., morphological analysis in light microscopy (LM) using phase contrast (PhC), SEM (Scanning Electron Microscopy) and DNA sequencing (see results for details).

Table 1. Sampling data

Sample	Locality	Coordinates	Collection date	Collector	Sample type
S1911	Calvert Island, Lookout	51.651144 - 128.138404	29/06/2021	Gillian Sadlier-Brown	Moss on ground
S2075	Vancouver Island, Mount Arrowsmith	49.233972 - 124.608917	08/08/2022	Kendrick Marr	Moss on tree bark
S2085	Calvert Island, Lookout	51.651144 - 128.1384038	23/06/2022	Kelly Fretwell	Moss on stone

Microscopy and Imaging

The specimens for LM were mounted on microscope slides in a small drop of Hoyer's medium, secured with a cover slip and dried at 60 °C for a week. The slides were examined with an Olympus BX53 or a Leica DM RB microscope (both with PhC) associated with an AmScope MU1803 digital camera, the figures were assembled in Adobe Photoshop CS4 Extended (Adobe System Corporation).

Morphometrics and morphological nomenclature

Structures were measured according to Kaczmarek et al. (2021) and only if their orientation was suitable. Body length was measured from the anterior extremity to the posterior end of the body, excluding the hind legs. The morphometric data were handled using the Echiniscoidea ver. 1.4 template available from the Tardigrada Register, http://tardigrada.net/register modified to include the claw spur insertion height (DeMilio et al. 2022). Raw measurements are available in cjz-2024-0040suppla and cjz-2024-0040supplb.

Genotyping

DNA was extracted from individual animals following the Chelex® 100 resin extraction method (BioRad) by Casquet et al. (2012) with modifications described in detail in Stec et al. (2020). Four DNA fragments, three nuclear (18S rRNA, 28S rRNA, ITS1) and one mitochondrial (COI) were sequenced. Fragments were amplified and sequenced according to the primers and protocols described in Stec et al. (2020) (18S rRNA, 28S rRNA, COI) and Gąsiorek (2022) (ITS1). Sequencing products were read with an ABI 3130xl sequencer at the Department of Biological and Environmental Sciences (University of Jyväskylä, Finland).

Phylogenetic reconstruction

The phylogenetic analyses were conducted using concatenated 18SrRNA+28SrRNA+COI+ITS1 sequences. The GenBank accession numbers of the newly generated sequences and those used in the phylogenetic reconstruction are presented in Table 2. Few *Diploechiniscus oihonnae* 18S rRNA sequences (GenBank accession numbers JX114910 and JX676181-6) and a 28S rRNA sequence (JX114869) were not considered as they did not overlap with the other sequences in the alignment. The 18S rRNA, 28S rRNA, and ITS1 sequences were aligned with MAFFT ver. 7 (Katoh 2002; Katoh and Toh 2008) with the G-INS-i method (thread=4, threadtb=5, threadit=0, reorder, adjustdirection, anysymbol, maxiterate=1000, retree 1, globalpair input). The COI sequences were aligned according to their amino acid sequences (translated using the invertebrate mitochondrial code) with the MUSCLE algorithm (Edgar 2004) in MEGA11 (Tamura et al. 2021) with default settings (all gap penalties=0, max iterations=8, clustering method=UPGMB, lambda=24). Alignments were visually inspected and trimmed in MEGA7. Sequences were concatenated with the R package 'concatipede' v1.0.0 (Vecchi and Bruneaux 2021). The Maximum Likelihood (ML) phylogenetic reconstruction was performed on the partitioned alignment with IQ-tree (Trifinopoulos et al. 2016) on the W-IQ-TREE online web server with default settings. For the Bayesian Inference (BI) phylogenetic reconstruction, model selection was performed for each alignment partition (six in total: 18S rRNA, 28S rRNA, ITS1 and three COI codons) with PartitionFinder2 (Lanfear et al. 2016). BI phylogenetic reconstruction was done with MrBayes v3.2.6 (Ronquist et al. 2012). Two runs with one cold chain and three heated chains were run for 50 million generations with a burning of 5 million generations, sampling a tree every 50000 generations. Posterior distribution sanity was checked with the Tracer v1.7 (Rambaut et al., 2018). MrBayes input file with the input alignment (and partitions models) is available as Supplementary Materials (cjz-2024-0040supplc). The phylogenetic trees were visualized with FigTree v1.4.4 (Rambaut 2007) and the image was edited with Inkscape 0.92.3 (Inkscape

Project 2020). The complete phylogenetic trees are available in cjz-2024-0040suppld and cjz-2024-0040supple.

Table 2. GenBank accession numbers of the sequences used in the phylogenetic reconstruction.

Tip name	SSU	LSU	COI	ITS1	References
Acanthechiniscus goedeni (Grigarick, Mihelčič & Schuster, 1964)	OP730 698	OP730 697	OP729 912		Vecchi et al. (2023*b*)
Antechiniscus lateromamillatus (Ramazzotti, 1964)	HM19 3370		HM19 3404		Jørgensen et al. (2011)
Barbaria bigranulata (Richters, 1907)	MZ820 792	MZ820 810	MZ820 846	MZ820 828	Gąsiorek et al. (2022)
Barbaria danieli (Meyer, Tsaliki & Sorgee, 2017)	MZ820 800	MZ820 818	MZ820 853	MZ820 836	Gąsiorek et al. (2022)
Barbaria madonnae (Michalczyk & Kaczmarek, 2006)	MZ820 803	MZ820 821	MZ820 854	MZ820 839	Gąsiorek et al. (2022)
Barbaria ollantaytamboensis (Nickel, Miller & Marley, 2001)	MZ820 804	MZ820 822	MZ820 855	MZ820 840	Gąsiorek et al. (2022)
Barbaria paucigranulata Wilamowski, Vončina, Gąsiorek & Michalczyk, 2022	MZ820 808	MZ820 826	MZ820 859	MZ820 844	Gąsiorek et al. (2022)
Barbaria weglarskae Gąsiorek, Wilamowski, Vončina & Michalczyk, 2022	MZ820 809	MZ820 827	MZ820 860	MZ820 845	Gąsiorek et al. (2022)
Bryodelphax australasiaticus Gąsiorek, Vončina, Degma & Michalczyk, 2020a	MT333 468	MT333 460		MT333 477	Gąsiorek et al. (2020*a*)
Bryodelphax decorates Gąsiorek, Vončina, Degma & Michalczyk, 2020a	MT333 469	MT333 462		MT333 478	Gąsiorek et al. (2020*a*)
Bryodelpha maculatus Gąsiorek, Stec, Morek, Marnissi & Michalczyk, 2017	MT333 471	MT333 464		MT333 479	Gąsiorek et al. (2020*a*)
Bryodelphax nigripunctatus Degma, Gąsiorek, Vončina & Michalczyk, 2020	MT333 472	MT333 465			Gąsiorek et al. (2020*a*)
Cornechiniscus cornutus (Richters, 1907)	MT420 869	MT420 853	MT420 439	MT420 860	Gąsiorek and Michalczyk, (2020)
Cornechiniscus imperfectus Gąsiorek & Michalczyk, 2020	MT420 872	MT420 856	MT420 451	MT420 865	Gąsiorek and Michalczyk, (2020)
Cornechiniscus madagascariesis Maucci, 1993	MT420 871	MT420 855	MT420 450	MT420 863	Gąsiorek and Michalczyk, (2020)
Cornechiniscus subcornutus Maucci & Ramazzotti, 1981	MT420 873	MT420 857	MT420 457	MT420 867	Gąsiorek and Michalczyk, (2020)
Diploechiniscus oihonnae 1 Type loc.	MT416 481	MT416 552	MT418 513		Kaczmarek et al. (2021)
Diploechiniscus oihonnae 2			MW71 5563		Morek et al. (2021)
Diploechiniscus oihonnae 3	MW13 6887	MW13 6923		MT374 201	Bochnak et al. (2020); Morek et al. (2021)
Diploechiniscus oihonnae 4			MG06 3724		Gąsiorek et al. (2017)
Diploechiniscus oihonnae 5			JX676 198		Vicente et al. (2013)
Diploechiniscus oihonnae 6			JX676 197		Vicente et al. (2013)
Diploechiniscus oihonnae 7			JX676 196		Vicente et al. (2013)
Diploechiniscus oihonnae 8			JX676 195		Vicente et al. (2013)
Diploechiniscus oihonnae 9			JX676 194		Vicente et al. (2013)
Diploechiniscus oihonnae 10			JX676 193		Vicente et al. (2013)
Diploechiniscus oihonnae 11			JX676 192		Vicente et al. (2013)
Diploechiniscus oihonnae 12			JX676 191		Vicente et al. (2013)
Echiniscus aonikenk Gąsiorek, Bochnak, Vončina & Michalczyk, 2021	MZ467 756	MZ467 762	MZ444 670	MZ467 821	Gąsiorek et al. (2021*a*)
Echiniscus azoricus Fontoura, Pilato & Lisi, 2008	MK52 9673	MK52 9701			Gąsiorek et al. (2019)
Echiniscus dariae Kaczmarek & Michalczyk, 2010	MK52 9677	MK52 9705			Gąsiorek et al. (2019)
Echiniscus insularis Gąsiorek, Vončina & Kiosya, 2021	MW18 0887	MW18 0879	MW17 8242	MW18 0910	Kiosya et al. (2021)

Echiniscus masculinus Gąsiorek, Vončina & Michalczyk, 2020	MT106 621	MT106 620	MT106 223	MT106 623	Gąsiorek et al. (2020*b*)
Echiniscus pellucidus Gąsiorek, Bochnak, Vončina & Michalczyk, 2021	MZ467 759	MZ467 765	MZ444 675	MZ467 828	Gąsiorek et al. (2021*a*)
Echiniscus succineus Gąsiorek & Vončina, 2019	MK67 5903	MK67 5914	MK64 9675	MT374 198	Bochnak et al. (2020)
Echiniscus tantulus Gąsiorek, Bochnak, Vončina & Kristensen, 2020	MT126 785	MT126 765	MT107 427	MT108 138	Bochnak et al. (2020)
Echiniscus testudo (Doyère, 1840)	MK52 9686	MK52 9716		MT374 199	Gąsiorek et al. (2019); Bochnak et al. (2020)
Hypechiniscus cataractus Gąsiorek, Oczkowski, Kristensen & Michalczyk, 2021	MT809 235	MT809 198		MT809 182	Gąsiorek et al. (2021*b*)
Hypechiniscus daedalus Gąsiorek, Oczkowski, Bartels, Nelson, Kristensen & Michalczyk, 2021	MT809 237	MT809 200		MT809 187	Gąsiorek et al. (2021*b*)
Hypechiniscus exarmatus (Murray, 1907)	MT809 238	MT809 201		MT809 190	Gąsiorek et al. (2021*b*)
Hypechiniscus flavus Gąsiorek, Oczkowski, Suzuki, Kristensen & Michalczyk, 2021	HM19 3377		HM19 3410		Jørgensen et al. (2011)
Hypechiniscus geminus Gąsiorek, Oczkowski, Suzuki, Kristensen & Michalczyk, 2021	HM19 3378		HM19 3411		Jørgensen et al. (2011)
Hypechiniscus gladiator (Murray, 1905)	MT809 243	MT809 203			Gąsiorek et al. (2021*b*)
Kristenseniscus tessellatus (Murray, 1910)	MK52 9690	MK52 9720			Gąsiorek et al. (2019)
Kristenseniscus walteri (Pilato & Lisi, 2003)	MK52 9691	MK52 9721			Gąsiorek et al. (2019)
Mopsechiniscus granulosus Mihelčič, 1967	HM19 3379		HM19 3412		Jørgensen et al. (2011)
Nebularmis auratus Gąsiorek & Michalczyk, 2021	MW18 0881	MW18 0904	MW17 8237	MW18 0893	Gąsiorek et al. (2021*c*)
Nebularmis burmensis Gąsiorek & Vončina, 2021	MW18 0882	MW18 0905	MW17 8238	MW18 0894	Gąsiorek et al. (2021*c*)
Nebularmis cirinoi (Binda & Pilato, 1993)	MK52 9692	MK52 9722			Gąsiorek et al. (2019)
Nebularmis indicus Gąsiorek, Ciosek & Michalczyk, 2021	MW18 0886	MW18 0909	MW17 8240	MW18 0897	Gąsiorek et al. (2021*c*)
Oreella chugachii Calloway, Miller, Johansson & Whiting, 2011	GU552 458				Calloway et al. (2011)
Oreella mollis Murray, 1910	EU266 962				Sands et al. (2008)
Parechiniscus chitonides Cuénot, 1926	HM19 3380		HM19 3413		Jørgensen et al. (2011)
Proechiniscus hanneae (Petersen, 1951)	HM19 3381		HM19 3414		Jørgensen et al. (2011)
Pseudechiniscus angelusalas Roszkowska, Grobys, Bartylak & Kaczmarek, 2020	MW03 2006	MW03 2096	MW03 1217	MW03 2186	Gąsiorek et al. (2021*f*)
Pseudechiniscus dreyeri Gąsiorek, Vončina, Kristensen & Michalczyk, 2021	OK048 615	OK048 633	OK047 278	OK048 646	Gąsiorek et al. (2021*d*)
Pseudechiniscus quadrilobatus Iharos, 1969	MW03 1946	MW03 2035	MW03 1178	MW03 2125	Gąsiorek et al. (2021*f*)
Pseudechiniscus shintai Vončina, Kristensen & Gąsiorek, 2020	MT645 084	MT645 082	MT644 270	MT645 086	Vončina et al. (2020)
Pseudechiniscus totoro Gąsiorek, Vončina, Kristensen & Michalczyk, 2021	OK048 616	OK048 635		OK048 648	Gąsiorek et al. (2021*d*)
Testechiniscus spitzbergensis spitzbergensis (Scourfield, 1897)	MH27 9664	MH28 6188		MH28 6189	Gasiorek et al. (2018*a*)
Testechiniscus spitzbergensis tropicalis Gąsiorek, Stec, Zawierucha, Kristensen & Michalczyk, 2018	MH27 9665	MH28 6187		MH28 6191	Gasiorek et al. (2018*a*)
Testechiniscus laterculus (Schuster, Grigarick & Toftner, 1980)	OQ029 311		OQ029 483		Massa et al. (2024)
Viridiscus aff. *viridianus*	MZ868 197	OK094 230	MZ852 064	OK094 211	Gąsiorek et al. (2021*e*)
Viridiscus perviridis (Ramazzotti, 1959)	MK52 9696	MK52 9726			Gąsiorek et al. (2021*e*)
Viridiscus viridissimus (Péterfi, 1956)	MZ868 194	OK094 225	MZ852 051	OK094 195	Gąsiorek et al. (2021*e*)
Diploechiniscus aff. *dimorphus* [Ca. 1 OR859978] S2010 v06	OQ029 306		OQ029 482		Massa et al. (2024)
Diploechiniscus aff. *dimorphus* [Ca. 1 OR859978] S2010 v03	OQ029 307				Massa et al. (2024)
Diploechiniscus aff. *dimorphus* [Ca. 1 OR859978] S2085 T4	**OR853 682**		**OR859 977**		**This study**

Diploechiniscus aff. *dimorphus* [Ca. 1 OR859978] S1911 Dpl1	**OR853 684**	**OR853 686**	**OR859 978**		**This study**
Diploechiniscus dimorphus **n. sp.** S1911 Dpl2	**OR853 683**	**OR853 687**	**OR859 979**		**This study**
Diploechiniscus dimorphus **n. sp.** S1911 Dpl5			**OR859 980**		**This study**
Diploechiniscus dimorphus **n. sp.** S1911 v1m			**OR859 981**	**OR853 688**	**This study**
Diploechiniscus dimorphus **n. sp.** S1911 v3m			**OR859 982**	**OR853 692**	**This study**
Diploechiniscus dimorphus **n.sp.** S1911 v4f			**OR859 983**	**OR853 693**	**This study**
Diploechiniscus dimorphus **n. sp.** S1911 5m			**OR859 984**	**OR853 690**	**This study**
Diploechiniscus dimorphus **n. sp.** S1911 v6f			**OR859 985**	**OR853 694**	**This study**
Diploechiniscus dimorphus **n. sp.** S1911 v7f			**OR859 986**	**OR853 689**	**This study**
Diploechiniscus dimorphus **n. sp.** S1911 v8f			**OR859 987**	**OR853 691**	**This study**
Diploechiniscus dimorphus **n. sp.** S1911 v11m			**OR859 988**	**OR853 695**	**This study**
Diploechiniscus horningi S2075 Dpl1	**OR853 685**		**OR859 976**		**This study**

Species delimitation analysis

A subset of the COI alignment (only sequences from *Diploechiniscus,* plus *Testechiniscus* Kristensen 1987 as outgroup) was used for species delimitation. Species delimitation was performed with ASAP (Puillandre et al., 2021) and bPTP (Zhang et al. 2013). ASAP species delimitation was performed on the p-distance matrix of the alignment on the ASAP online server (Puillandre et al. 2021) with default parameters (complete results are available as cjz-2024-0040supplaf). bPTP species delimitation was performed on the bPTP online server with default parameters using as input a phylogenetic tree obtained from the COI reduced alignment following the same pipeline as above (complete results are available as cjz-2024-0040supplg). Additionally, p-distances between the sequences used in the species delimitation analyses were calculated with MEGA11 (with pairwise deletion) (Tamura et al. 2021) (cjz-2024-0040supplh).

Other comparative material

Slides with *Echiniscus* cf. *robertsi* (Slides S2010_SL1 – C5084s1 and S2010.Ech.V03-6 – C5084v1-2) and *Diploechiniscus* sp. (Slide S2013_SL1 – C5087s1) (Massa et al. 2024) from Haida Gwaii Island (British Columbia) were examined.

Results

Morphology

From samples S1911 and S2085, Echiniscidae with constant dorsal plates ornamentation (described in detail below in the taxonomic account section) but variable chaetotaxy were identified. The individuals were divided in six morphotypes based on their chaetotaxy and claw types: α) Individuals with only dorsolateral spicules D; β) Individuals with only lateral filaments D; γ) Individuals with both dorsolateral spicules D and lateral filaments D; δ) Individuals with lateral filaments in B (not always), C and D; ε) Individuals without appendages (other than in A) and four claws per leg; ζ) Individuals without appendages (other

than in A) and two claws per leg. The most common morphotypes were α and β, whereas the others were less represented (Table 3). All the sequenced specimens with α morphotype resulted to be females, whereas those with the β morphotypes were males.

Table 3. Number of individuals found for each main morphotype in samples S1911 and S2085 (individuals mounted on stubs for SEM are not included in this count). The letters in parenthesis refer to the chaetotaxy of the specimens.

Morphotype (chaetotaxy)	Females	Males	Juveniles*	Undetermined
α (A-D^d)	19		2	
β (A-D^l)		27	1	
γ (A-D^d-D^l)		1	1	4
δ (A-(B^l)-C^l-D^l)	2	2		
ε (A)				8
ζ (A)°			2	

* Individuals without gonopore; ° this morphotype corresponds to 1st instar (or 2-clawed) larvae.

DNA extraction, phylogenetic reconstruction and species delimitation

The DNA extraction and amplification was successful for 13 individuals (11 from sample S1911, one from sample S2085, one from sample S2075). However, the sequencing, recovery and mounting of carcasses were only successful for eight individuals from the α and β morphotypes from sample S1911 and for the individual from S2075.

The phylogenetic reconstructions (Fig. 1) recovered the Echiniscidae from S1911 and S2085 in the *Diploechiniscus* clade together *D. oihonnae* and *D. horningi* and with *Diploechiniscus* specimens previously sequenced by Massa et al. (2024) (*Echiniscus* cf. *robertsi*; sample S2010). Specimens identified as *D. horningi* (from sample S2075 – see *Diploechiniscus horningi* section below) were recovered as separate species by both ASAP and bPTP species delimitation analyses.

The newly analyzed specimens of *Diploechiniscus* belonged to three distinct clades, A, B, and D; Fig. 1): A and B clades were sister groups to each other, while D (*D. horningi* specimen) was sister group of clade C (*D. oihonnae* specimens). *Diploechiniscus* of clades A (highly supported) and B were recovered as separate species by bPTP and ASAP, however both methods recover one individual from clade A (S1911-5m) as a different species, however this was probably not reliable as this individual COI distance with the other members of clade A was on average ~2.42% (cjz-2024-0040supplh). Clade A includes two sequences from GenBank (S2010-v03 and S2010-v06) (from Massa et al. 2024) for which a voucher of the carcass is available, and originally classified as *Echiniscus* cf. *robertsi* (Massa et al. 2024).

Diploechiniscus horningi

Two individuals from sample S2075 were attributed to *Diploechiniscus horningi* based on their morphology and previous records of this species on Vancouver Island (Kathman and Dastych 1990) (the same island from which sample S2075 came from). One individual was used for DNA extraction and sequencing and the carcass was recovered and mounted on slide, whereas the second animal was mounted on slide. Both the phylogenetic reconstruction (Fig. 1) and the

species delimitation analyses (Fig. 1) indicated that *D. horningi* is a separate species respect to the other species of *Diploechiniscus* including *D. oihonnae*.

Discussion

We identified members of the genus *Diploechiniscus* which deviate from the typical morphology of the genus by having fewer number of filaments and spines, a less evident double sculpture of the dorsal plates and not having visible black eyes in mounted specimens (black eyes are visible in mounted specimens in *D. oihonnae*) (Vicente et al. 2013; Kaczmarek et al. 2021; Gąsiorek 2023). However, these individuals are confidently assigned to this genus based on their phylogenetic position and other morphological characters (see taxonomic account). We are temporarily refraining from erecting a new genus for these animals due to the lack of evident morphological traits that would allow its clear definition. However, this remains a possibility in the future should more populations be identified, allowing for a much more detailed morphological analysis.

Two *Diploechiniscus* species corresponding to clade A and B (Fig. 1), which co-occur in one particular sample (S1911), are present based on COI species delimitation; however, due to the few issues explained below, we can confidently describe only one of them (clade A) as new to science, naming it *Diploechiniscus dimorphus* n. sp.(see taxonomic account).

For *D. dimorphus* n. sp. (clade A), we managed to obtain a good number of voucher specimens linked to COI sequences, of both males and females. These individuals show a striking sex dimorphism related to chaetotaxy (females with α morphotype and males with β morphotype; Tab. 3). For the clade B, we could not obtain new informative voucher specimens linked to COI sequences. Given that these two *Diploechiniscus* species can be mixed in a sample (i.e. S1911), it is challenging to morphologically differentiate the two species (clades A and B) with present available information. However, Fig. 1 shows that specimens of S1911 and S2085 samples of clade B are conspecific with two individuals from Haida Gwaii (sample S2010; Massa et al. 2024) for which vouchers are available (even if not in great condition). The Haida Gwaii individuals was found together with four other individuals with identical morphology (Massa et al. 2024), thus it is possible to use these individuals to obtain reliable information on the *Diploechiniscus* of clade B. Reanalysis of the specimens from Haida Gwaii (sample S2010) showed that both males and females have the β morphotype, thus allowing to discriminate between the two species (clades A and B) based on the females morphology only. There are insufficient specimens and data to formally describe a new species from specimens of clade B (4 individuals and 2 carcasses from Haida Gwaii; Massa et al. 2024), although the molecular data clearly identified it as separate clade; for these reasons we identify this clade as Unconfirmed Candidate Species (UCS) naming it as *Diploechiniscus* aff. *dimorphus* [Ca1 OR859978] (also reported as *Echiniscus* cf. *robertsi* in Massa et al. 2024).

From individuals of all the other morphotypes (γ, δ, ε) we could not get DNA sequences, and as they are present in low number, for the moment we do not consider them in the taxonomic description. Those specimens probably represent examples of intraspecific variability in A and/or B as variability in chaetotaxy in *Diploechiniscus* is known to occur (Kaczmarek et al. 2021; Gąsiorek 2023). In not considering them, we are aware that we are underestimating the variability of the new species. However, we prefer to be cautious pending analysis of more specimens (measurements of those individuals not assigned to the new species are available as

cjz-2024-0040supplb). As the males of clades A and B are indistinguishable and the proportion of animals of clade B is low (1 over 11 individuals) in sample S1911 (where all animals of clade A are present) and based on DNA data, the morphological description of the males (and juveniles/second stage larvae) of *Diploechiniscus dimorphus* n. sp. was made including all the males from sample S1911. The morphotype ζ, corresponding to first stage larvae, it tentatively attributed to *D. dimorphus* n. sp. because the specimens of this species are more abundant in the sample (as evidenced by the molecular data), therefore the two first stage larvae found very probably correspond to this species.

We showed that within *Diploechiniscus* there is a species (that we identified with *D. horningi*) morphologically indistinguishable from *D. oihonnae* but well distinct from it based on molecular data (both the phylogenetic reconstruction and the species delimitation analyses confirm these differences, and the COI p-distances of the *D. horningi* to *D. oihonnae* individuals range from 13 to 15%). Kaczmarek et al. (2021) synonymized *Diploechiniscus horningi* to *D. oihonnae* based on morphological characters. In light of our results, together with the previous record of *D. horningi* in our sampling site (Mount Arrowsmith, Vancouver Island; Kathman and Dastych 1990) and the relatively close geographical proximity of our sampling site to the type locality of the species in the Pacific Northwest (i.e. Silver Falls State Park, OR, USA; Schuster and Grigarick 1971), we propose the reinstatement of *D. horningi* as a valid species.

The presence of bisexual species in Echiniscidae is relatively rare, and generally there are no evident sexual dimorphic characters between males and females, other than the shape of the gonopore ("six-petal rosette" around the pore in female, crescent-shaped pore at the extremity of a short papilla-like structure in male). In the family, other common differences among sexes are related to not so evident phenotypic characters as body size (larger in female, in relation to the development of larger gonads; Guidetti et al. 2007) and/or slightly differences in morphometry of some structures, as claws, cephalic and trunk appendages (e.g. Dastych 1987; Kristensen 1987; Claxton 1996, 2001; Guidetti et al. 2007; Mitchell and Romano 2007; Rebecchi et al. 2008; Gąsiorek et al. 2018*a*,*b*; Cesari et al. 2020; for a more information on sexual dimorphism in the Echiniscidae see also Gasiorek et al. 2020).

Other sexual dimorphic characters are extremely rare. Differences in chaetotaxy between sexes, as we found in *D. dimorphus* n. sp., have been previously recorded in only three species among the 14 genera of Echiniscoidea with dioecious species. In particular, *Echiniscus masculinus* Gąsiorek, Vončina and Michalczyk 2020 females have a D^d spine which is absent in male, *Antechiniscus parvisetus* (Horning and Schuster 1983) females have a spine in D replaced by a filament in males (Claxton 2001) and *Antechiniscus moscali* Claxton 2001 females have small triangular spines in B, C and D often missing in males.

Within *Diploechiniscus, D. oihonnae* shows very reduced and not easily detectable morphological differences between males and females, i.e. males are slender than females and have a larger granulation of the genital plates than in other parts of ventral side, and there are morphometric differences in few cuticular structures between sexes (Kaczmarek et al. 2021). The new *Diploechiniscus* species challenges the morphological homogeneity of *Diploechiniscus* as currently defined, indicating that much work is still needed to have a clear and complete comprehension of armored tardigrades evolution and variability. The differences in chaetotaxy in males and females of *D. dimorphus* n. sp., show that caution should be applied

when using chaetotaxy as a diagnostic trait in species differentiation and classification due to intraspecific variation.

Taxonomic account

Diploechiniscus dimorphus n. sp.

Diploechiniscus sp. in Vecchi et al. (2022*b*)
Diploechiniscus sp. in Massa et al. (2024)

urn:lsid:zoobank.org:act:12D4E509-1F06-44ED-B2EC-0BA9A7991ECA

Figures 2-5, Tables 4-6, cjz-2024-0040suppla
Type locality: Lookout, Calvert Island, British Columbia, Canada. 51.651144 -128.138404.
Etymology: The specific epithet comes from the pronounced sexual dimorphism of this species.
Type material: Holotype and 50 paratypes (on slides): two specimens of the 1st instar larvae (no anus, no gonopore, two claws per leg); three specimens of 2nd instar larvae (anus, no gonopore, four claws per leg); 45 adults (gonopore present, four claws per leg), of which 27 identified as males and 18 identified as females. In addition, 16 paratypes (six females, seven males, two 2nd stage larvae, one 1st stage larvae) on stub for SEM observation.
Type depositories: The holotype (S1911-C5010.s17) and four paratypes are deposited in the Tardigrada collection of the Natural History Museum of Verona, Italy (Catalogue codes of slides C5010-s22,-s24,-s25,-s53), nine paratypes in Bertolani collection, University of Modena and Reggio Emilia, Modena, Italy (Catalogue codes of slides C5010s1,-s31,-s35,-s38,-s61,-V4,-V6,-V7,-V8), four paratypes are deposited in the tardigrade collection of the Institute of Systematics and Evolution of Animals (Polish Academy) of Sciences, Kraków, Poland (Catalogue codes of slides CA.075.01 – 6), and six paratypes are deposited in the Invertebrate Zoology Collections of the Royal BC Museum, Victoria, Canada (Catalogue codes of slides 024-00019-001 – 4).

Description

Animals (measurements and statistics in Tables 4-6)
Females. (measurements and statistics in Table 4). Body orange. Eyes not visible in animals mounted in Hoyer's medium. Complete set of head appendages present: internal and external cirri; large, drop-shaped cephalic papillae; cirrus A; large, finger-like clava near the base of cirrus A (Figs. 2A-2B). Cuticle on dorsal plates appears constituted by polygonal granules (i.e. head of epicuticular pillars; about 1-1.5 µm in diameter), larger in dorsal-central portion of the body (about 1 µm) (Fig. 2A); each granule generally surrounded by five to seven other granules (Fig. 3A); in the scapular and terminal plates of several animals these granules are connected by thin line (striae). A line of pores (that appear in LM-PhC as white circle of about 0.8 µm above the polygonal dark granules) is present along the posterior and anterior margins of the scapular plate (aka dorsal plate I) and anterior margin of terminal plate, few other pores are

scattered on each dorsal plate (Figs. 3A, 4E). First median plate (m1) triangular (Figs. 2A, 3A, 4A). Second median plate (m2) rhomboidal, divided into two triangular parts by a smooth stripe without sculpture: the sculpture of the anterior part not visible with LM, except for a thin anterior band, the sculpture of the posterior part always visible with LM (Figs. 2A-B, 3A). Third median plate (m3) triangular and the smallest of the three (Figs. 2A, 3A, 4A). First and second paired plates (aka dorsal plate II and III) similar in size are divided into two parts, a narrower anterior part and a wider posterior part, by a smooth stripe without sculpture (Figs. 2A, 3A, 4A). Dorsal appendages as small triangular teeth of variable size (about 1-3 µm long) in D^d position (they can be present in only one side or rarely be absent) (Figs. 2A, 4A, 4G). Terminal plate (aka dorsal plate IV) faceted and with two posterior notches bearing very small dorsolateral spines on their margins, corresponding to E' position (they can be present in only one side) (Figs. 2A-B, 4A, 4G). With SEM, in correspondence with the curvature of the terminal plate, depressions (pores) of the same size as the polygons of the dorsal sculpture are visible (Fig. 4G). Lateral appendages are absent (Figs. 2A-B). Buccopharyngeal apparatus typical of the genus, dissolved in most mounted specimens; only the most anterior portion of the buccal tube and the cuticular bars within the pharynx remained visible in several individuals. Between the dorsal plates there are areas with small polygonal granules or dots (Fig. 3A). Gonopore rosette-shaped, anterior to the anus (Figs. 3C, 4B, 4D). With LM, lateral and ventral surface of the body with small dots, larger in the gonopore area (in correspondence of the genital plates) (Figs. 3C, 3E). With SEM, the dots of the lateral and ventral surface correspond to the heads of the cuticular pillars, which appear as uniformly distributed granules bearing small central depressions or, if the cuticle is tight due to the "pop-corn" effect related to SEM preparation (see Camarda et al., 2023), only the small depressions are visible (Fig. 5B). With SEM, two poorly marked rugose areas are present below the head, and two other, more marked, rugose areas are present beside the gonopore; these areas can be interpreted as not well defined plates (i.e. subcephalic and genital plates, respectively) (Figs. 4C-D).

All legs are covered with dots/granules (visible in LM), except the middle external surface in which a rugose area is present, which can be interpreted as not well defined plate (Figs 3C). Dentate collar on legs IV with fine granulation and about four-six large teeth (teeth usually with a single point and slightly irregular in size) (Fig. 2B). Triangular spine on leg I and finger-like papilla on leg IV present (Fig. 3C). External claws of all legs smooth, internal claws with spurs directed downwards; spurs are larger on the claws of the hind legs (Figs 3C, 3E, 4D, 4F).

Table 4. Measurements of selected morphological structures of adult females of *Diploechiniscus dimorphus* n. sp. 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; sp – the proportion between the length of a given structure and the length of the scapular plate.

CHARACTER	**N**	**RANGE**		**MEAN**		**SD**		**Holotype**	
		µm	***sp***	**µm**	***sp***	**µm**	***sp***	**µm**	***sp***
Body length	7	129 – 159	*435 – 570*	148	*515*	10	*53*	144	*542*
Scapular plate length	11	23.2 – 33.3	–	28.1	–	2.8	–	26.6	–
Head appendages lengths									

Cirrus *internus*	11	7.7	–	14.5	*30.1*	–	*52.3*	11.8	*42.0*	2.3	*7.9*	13.1	*49.0*
Cephalic papilla	11	3.6	–	6.0	*15.5*	–	*20.1*	5.0	*17.7*	0.7	*1.5*	5.3	*20.1*
Cirrus *externus*	10	8.4	–	15.8	*36.2*	–	*57.7*	13.2	*47.0*	2.5	*7.6*	15.4	*57.7*
Clava	8	4.1	–	5.1	*13.1*	–	*18.9*	4.7	*16.4*	0.4	*2.0*	5.0	*18.9*
Cirrus *A*	9	46.2	–	72.0	*164.8*	–	*259.1*	57.0	*203.0*	7.3	*25.9*	54.9	*206.3*
Cirrus *A*/Body length ratio	6	36%	–	45%		–		41%	–	4%	–	38%	–
Body appendages lengths													
Cirrus D^d	6	1.5	–	3.2	*5.4*	–	*9.6*	2.0	*6.9*	0.6	*1.6*	1.6	*5.9*
Cirrus *E*	1	1.7	–	1.7	*6.3*	–	*6.3*	1.7	*6.3*	?	*?*	?	*?*
Spine on leg I length	5	2.3	–	3.1	*8.0*	–	*11.2*	2.7	*9.0*	0.3	*1.3*	?	*?*
Papilla on leg IV length	7	2.9	–	3.9	*8.7*	–	*14.0*	3.2	*11.3*	0.4	*1.6*	3.1	*11.6*
Number of teeth on the collar	7	5	–	7		–		5.9	–	0.7	–	7	–
Claw I heights													
Branch	7	7.5	–	8.3	*24.4*	–	*30.0*	7.8	*27.3*	0.3	*2.0*	7.8	*29.1*
Spur insertion	7	2.1	–	2.4	*6.6*	–	*8.2*	2.2	*7.6*	0.1	*0.6*	2.1	*8.0*
Spur	7	1.5	–	2.1	*5.5*	–	*7.6*	1.8	*6.4*	0.2	*0.8*	1.5	*5.7*
Spur/branch height ratio	7	20%	–	25%		–		23%	–	2%	–	20%	–
Claw II heights													
Branch	7	6.5	–	7.8	*23.5*	–	*28.1*	7.4	*25.8*	0.4	*1.9*	7.4	*27.9*
Spur insertion	7	1.6	–	2.1	*5.5*	–	*7.8*	1.8	*6.4*	0.2	*0.9*	2.1	*7.8*
Spur	7	1.4	–	1.8	*4.7*	–	*6.3*	1.6	*5.5*	0.1	*0.5*	1.4	*5.3*
Spur/branch height ratio	7	19%	–	23%		–		21%	–	1%	–	19%	–
Claw III heights													
Branch	7	6.8	–	7.9	*23.8*	–	*28.6*	7.4	*25.9*	0.4	*1.9*	7.5	*28.0*
Spur insertion	6	1.5	–	2.0	*5.4*	–	*7.3*	1.8	*6.2*	0.2	*0.7*	?	*?*
Spur	5	1.5	–	1.8	*4.4*	–	*6.3*	1.6	*5.5*	0.1	*0.7*	?	*?*
Spur/branch height ratio	5	19%	–	23%		–		21%	–	2%	–	?	–
Claw IV heights													
Branch	7	7.7	–	9.1	*25.8*	–	*33.2*	8.5	*29.6*	0.5	*2.7*	8.9	*33.2*
Spur insertion	6	2.2	–	2.7	*7.1*	–	*9.7*	2.5	*8.7*	0.2	*1.1*	?	*?*
Spur	6	1.7	–	2.3	*6.3*	–	*8.1*	2.0	*6.9*	0.2	*0.6*	?	*?*
Spur/branch height ratio	6	22%	–	26%		–		24%	–	1%	–	?	–

Males. (measurements and statistics in Table 5). The males (Figs. 2C-D, 3B, 3D, 3F, 5) are similar to females, there are differences in the shape of the gonopore (short papilla-like structure; Figs 3D, 5E), in the presence of proportionally larger cephalic papillae (Figs 2C, 3F) and larger rugose areas (i.e. genital plates; Figs 3D, 5E) beside the gonopore, and in chaetotaxy (Figs 2C-D, 3B). Males have long lateral filament (or long spine) in D position (absent in females) and generally (but not always) lack the small spines in the E' position of terminal plate (Figs 2C, 2D).

Table 5. Measurements of selected morphological structures of adult males of *Diploechiniscus dimorphus* n. sp. 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; sp – the proportion between the length of a given structure and the length of the scapular plate.

CHARACTER	N	RANGE						MEAN		SD	
		µm			*sp*			µm	*sp*	µm	*sp*
Body length	12	112	–	145	*442*	–	*595*	129	*526*	11	*41*
Scapular plate length	16	20.3	–	32.5		–		25.1	–	2.7	–
Head appendages lengths											
Cirrus *internus*	15	10.8	–	22.1	*33.3*	–	*83.6*	16.0	*64.8*	3.0	*13.8*
Cephalic papilla	13	5.8	–	9.7	*18.0*	–	*40.2*	7.7	*31.3*	1.1	*6.3*
Cirrus *externus*	15	12.2	–	21.6	*44.7*	–	*87.9*	17.0	*68.5*	2.6	*11.2*
Clava	15	4.8	–	8.8	*14.8*	–	*34.5*	6.8	*27.4*	1.1	*5.4*
Cirrus *A*	13	39.7	–	64.2	*175.9*	–	*229.2*	51.3	*202.8*	6.9	*17.5*
Cirrus *A*/Body length ratio	10	34%	–	49%		–		39%	–	5%	–
Body appendages lengths	0										
Cirrus *D*	12	23.9	–	36.9	*112.8*	–	*149.8*	30.5	*124.6*	3.4	*12.8*
Spine on leg I length	5	1.5	–	2.9	*5.7*	–	*12.5*	2.2	*9.2*	0.5	*2.6*
Papilla on leg IV length	12	2.2	–	4.1	*7.8*	–	*16.5*	3.0	*12.4*	0.5	*2.2*
Number of teeth on the collar	12	4	–	6		–		5.1	–	0.7	–
Claw I heights											
Branch	12	6.9	–	8.0	*24.7*	–	*33.8*	7.4	*30.4*	0.3	*2.5*
Spur insertion	11	1.6	–	2.2	*6.5*	–	*10.2*	1.9	*7.9*	0.2	*1.0*
Spur	10	1.6	–	1.8	*6.1*	–	*8.2*	1.7	*7.0*	0.1	*0.7*
Spur/branch height ratio	10	21%	–	25%		–		23%	–	1%	–
Claw II heights											
Branch	12	6.6	–	7.7	*24.3*	–	*32.4*	7.1	*29.3*	0.3	*2.3*
Spur insertion	11	1.6	–	2.1	*6.4*	–	*8.9*	1.7	*7.1*	0.2	*0.7*
Spur	10	1.3	–	1.7	*5.4*	–	*6.6*	1.5	*6.1*	0.1	*0.4*
Spur/branch height ratio	10	20%	–	24%		–		21%	–	1%	–
Claw III heights											
Branch	12	6.3	–	7.7	*24.9*	–	*32.0*	7.1	*29.0*	0.4	*2.0*
Spur insertion	12	1.5	–	2.0	*6.2*	–	*7.6*	1.7	*6.9*	0.1	*0.4*
Spur	11	1.2	–	1.7	*5.3*	–	*6.9*	1.5	*6.0*	0.1	*0.5*
Spur/branch height ratio	11	17%	–	23%		–		21%	–	2%	–
Claw IV heights											
Branch	9	7.6	–	9.5	*27.5*	–	*38.3*	8.5	*34.9*	0.7	*3.2*
Spur insertion	8	2.3	–	2.9	*9.3*	–	*11.9*	2.5	*10.3*	0.2	*1.0*
Spur	7	2.0	–	2.1	*7.4*	–	*10.1*	2.0	*8.5*	0.1	*1.0*
Spur/branch height ratio	7	21%	–	27%		–		24%	–	2%	–

Larvae. (measurements and statistics in Table 6). Specimens with four claws per leg and without gonopore (2nd stage larva according to Bertolani et al., 1984) were found and attributed to this species due to the large number of specimens of this species respect to those of the clade A in the sample. The larvae present the same characters of the adults but with two types of chaetotaxy. One type with lateral filament in D position (ascribable to juvenile males), the other with small dorsal spine in D^d position and very small spine on the margins of the notches of terminal plate (ascribable to juvenile females). Two 2nd stage larvae were found with very small spines in D^d and filaments in D. Three 1st stage larvae (according to Bertolani et al., 1984) (72-82 µm long) with two claws per leg and without gonopore were found, showing no dorsal or lateral appendages.

Eggs. Not found.

Distribution: The species has been found only in Canada; other than the type locality (Calvert Island, Lookout, British Columbia), it was recorded also in Graham Island (Haida Gwaii, British Columbia) (Massa et al. 2024).

Table 6. Measurements of selected morphological structures of juveniles (2nd stage larvae) of *Diploechiniscus dimorphus* n. sp. 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; sp – the proportion between the length of a given structure and the length of the scapular plate.

CHARACTER	N	RANGE		MEAN		SD	
		µm	*sp*	µm	*sp*	µm	*sp*
Body length	2	133 – 147	*509 – 529*	140	*519*	10	*14*
Scapular plate length	2	25.1 – 28.8	–	26.9	–	2.6	–
Head appendages lengths							
Cirrus *internus*	2	14.2 – 18.2	*49.3 – 72.5*	16.2	*60.9*	2.9	*16.4*
Cephalic papilla	2	5.6 – 8.7	*19.4 – 34.6*	7.1	*27.0*	2.2	*10.7*
Cirrus *externus*	2	13.0 – 18.2	*45.3 – 72.6*	15.6	*58.9*	3.7	*19.3*
Clava	2	4.5 – 7.6	*15.7 – 30.1*	6.0	*22.9*	2.1	*10.1*
Cirrus *A*	2	44.1 – 64.8	*175.5 – 225.1*	54.4	*200.3*	14.6	*35.0*
Cirrus *A*/Body length ratio	2	33% – 44%	–	39%	–	8%	–
Body appendages lengths							
Cirrus *D*	1	28.1 – 28.1	*112.0 – 112.0*	28.1	*112.0*		
Cirrus D^d	1	3.4 – 3.4	*11.6 – 11.6*	3.4	*11.6*		
Spine on leg I length	1	2.7 – 2.7	*9.4 – 9.4*	2.7	*9.4*		
Papilla on leg IV length	2	2.7 – 3.5	*10.7 – 12.1*	3.1	*11.4*	0.6	*1.0*
Number of teeth on the collar	2	5 – 5	–	5.0	–	0.0	–
Claw I heights							
Branch	2	7.3 – 7.5	*26.1 – 29.1*	7.4	*27.6*	0.1	*2.1*

Spur insertion	2	2.1	–	2.2	*7.5*	–	*8.2*	2.1	*7.8*	0.1	*0.5*
Spur	2	1.8	–	1.8	*6.2*	–	*7.0*	1.8	*6.6*	0.0	*0.6*
Spur/branch height ratio	2	24%	–	24%		–		24%	–	0%	–
Claw II heights											
Branch	2	6.6	–	7.2	*25.1*	–	*26.4*	6.9	*25.7*	0.4	*0.9*
Spur insertion	2	1.6	–	1.8	*6.2*	–	*6.3*	1.7	*6.2*	0.1	*0.0*
Spur	2	1.3	–	1.5	*5.1*	–	*5.1*	1.4	*5.1*	0.1	*0.0*
Spur/branch height ratio	2	19%	–	20%		–		20%	–	1%	–
Claw III heights											
Branch	2	6.5	–	7.1	*24.6*	–	*26.0*	6.8	*25.3*	0.4	*1.0*
Spur insertion	1	1.7	–	1.7	*5.8*	–	*5.8*	1.7	*5.8*		
Spur	1	1.5	–	1.5	*5.1*	–	*5.1*	1.5	*5.1*		
Spur/branch height ratio	1	21%	–	21%		–		21%	–		–
Claw IV heights											
Branch	2	7.8	–	8.6	*30.0*	–	*31.1*	8.2	*30.6*	0.6	*0.8*
Spur insertion	1	1.9	–	1.9	*7.4*	–	*7.4*	1.9	*7.4*		
Spur	1	1.9	–	1.9	*7.4*	–	*7.4*	1.9	*7.4*		
Spur/branch height ratio	1	24%	–	24%		–		24%	–		–

Remarks

A small number of individuals (10 in total) with plates ornamentation compatible with *Diploechiniscus dimorphus* n. sp., but atypical chaetotaxy were found in the same samples (A-D^d-D^l; A-(B^l)-C^l-D^l; A). Due to the absence of genetic data for these morphologies, they were not included in the species description for precaution (see Discussion). Measurements of those individuals are available in cjz-2024-0040supplb.

Differential diagnosis.

The new species differs from:

- *Diploechiniscus oihonnae* (Richters 1903), based on the redescription of Kaczmarek et al. (2021), by several characters, in particular it differs for the very reduced number and size of the body appendages (e.g. in female absence of lateral appendages in B, C, D, and E, dorsal appendages in C, and dorsolateral spines in B, C and D), the lower number and not uniformly distributed cuticular pores, the fewer and larger teeth in the dentate collar, the absence of spurs in the external claws IV. *Diploechiniscus horningi* is considered by Kaczmarek et al. (2021) as morphologically indistinguishable from *Diploechiniscus oihonnae.*
- *Diploechiniscus laterosetosus* (Ito 1993) by several characters; in particular it differs for the very reduced number and size of the body appendages (e.g. in female absence of lateral appendages in B, C, D, and E, dorsal appendages in C, and dorsolateral spines in B, C and D), the lower number and not uniformly distributed cuticular pores, the absence of spurs in the external claws IV.
- *Diploechiniscus polygonalis* (Ito 1993) by several characters; in particular it differs for the very reduced number and size of the body appendages (e.g. in female absence of

lateral appendages in B, C, D, and E, dorsal appendages in C, and dorsolateral spines in B, C and D), the lower number and not uniformly distributed cuticular pores, the absence of spurs in the external claws IV.

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Competing interests

The authors declare there are no competing interests.

Author contribution statement

Conceptualization: MV, RG, HC
Data curation: MV, RG, JV
Formal analysis: MV, RG
Funding acquisition: SC
Investigation: MV, RG, JV
Methodology: MV, RG, JV
Project administration: RG, HC, SC
Resources: RG, HC, SC
Software: MV
Supervision: MV, RG
Validation: MV, RG, JV, HC, SC
Visualization: MV, RG
Writing – original draft: MV, RG
Writing – review and editing: MV, RG, JV, HC, SC

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This work was supported by the Academy of Finland Fellowship to SC (#314219 and #335759).

Data availability statement

Genetic data generated and analyzed during this study are available in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) repository (see main text for GenBank accession

numbers). Morphometric data generated and analyzed during this study are available and are provided in full within the published article and its supplementary materials.

Figures captions

Figure 1: Maximum likelihood phylogenetic reconstruction of *Diploechiniscus* and related genera, along with species delimitation analyses results. (*: hologneophore voucher from this study, **: hologenophore voucher from Massa et al. 2024). Individual S2010v03 was not included in species delimitation analyses as COI sequence is not available. Scale bar indicates substitutions/site. Number above nodes indicate ML/BI support values. ● indicates support values of 100/1. Shaded boxes indicate the identified *Diploechiniscus* species based on molecular and morphological data combined.

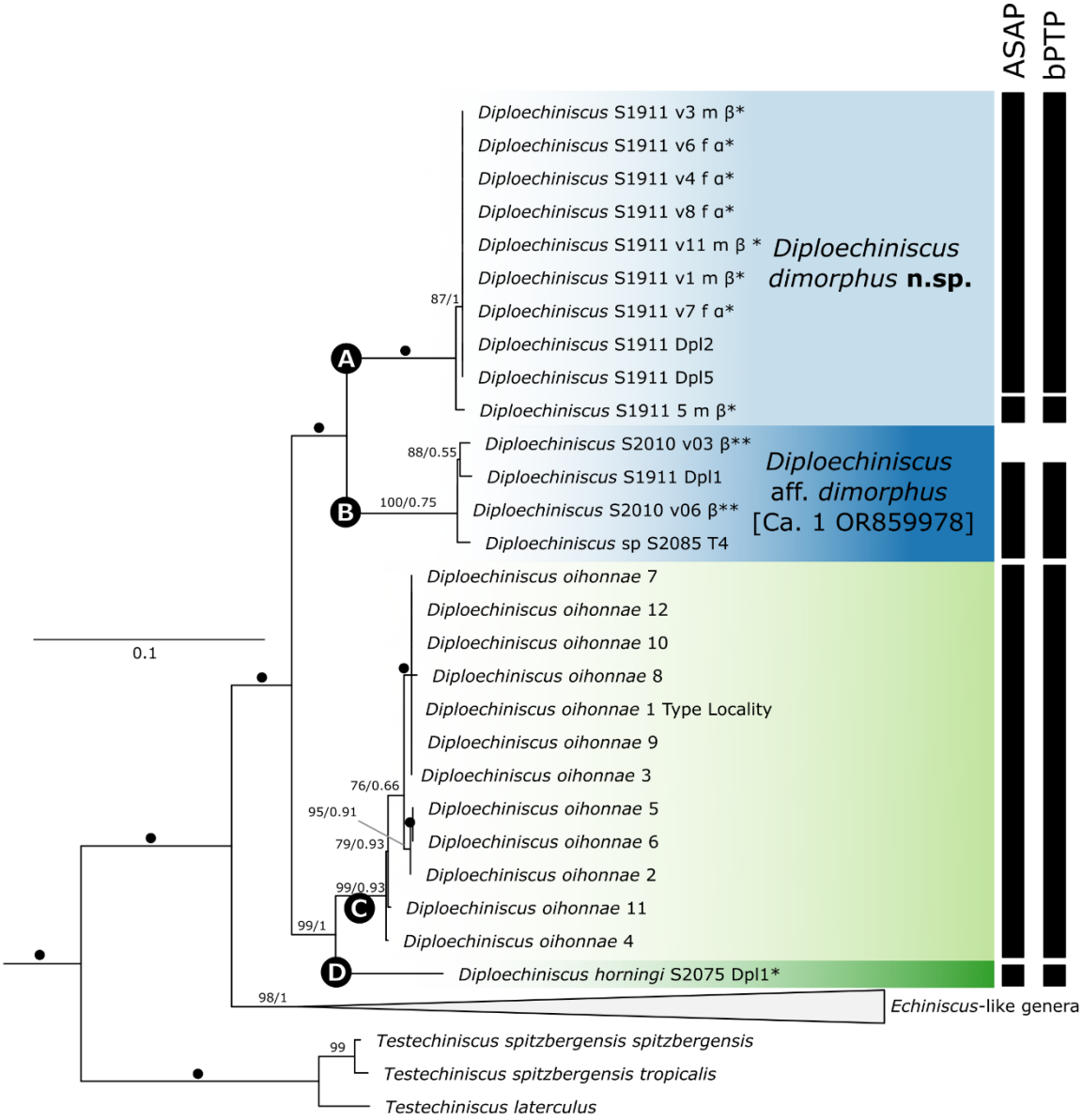


Figure 2. *Diploechiniscus dimorphus* n.sp. (LM). A. Female (holotype), dorsal view (arrowhead: small D^d spine). B. Female, lateral view (white arrowhead: small D^d spine; black arrowhead: small E' spine). C. Male, dorsal view. D. Male, lateral view. Scale bars: 20 µm.

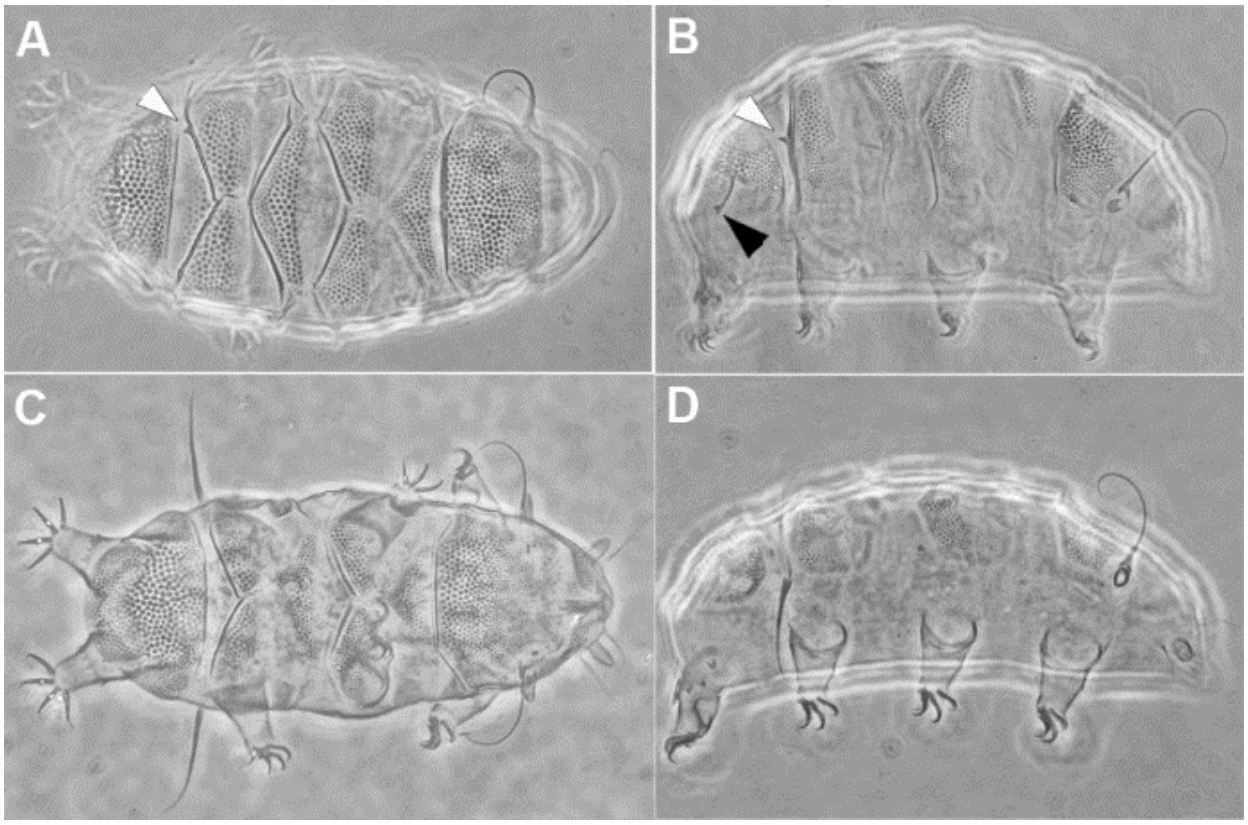


Figure 3. *Diploechiniscus dimorphus* n.sp. (LM). A. Female, dorsal view (arrows: holes in the cuticle; arrowhead: small D^d spine). B. Male, dorsal view (arrow: hole in the cuticle). C. Female gonopore (arrowhead), genital plates (asterisks). D. Male gonopore (arrowhead), genital plates (asterisks). E. Ventral surface of a female head cuticle with small dots. F. Head of a male with eyes and cephalic sensory organs. Scale bars: A-B = 20 µm, C-F = 10 µm.

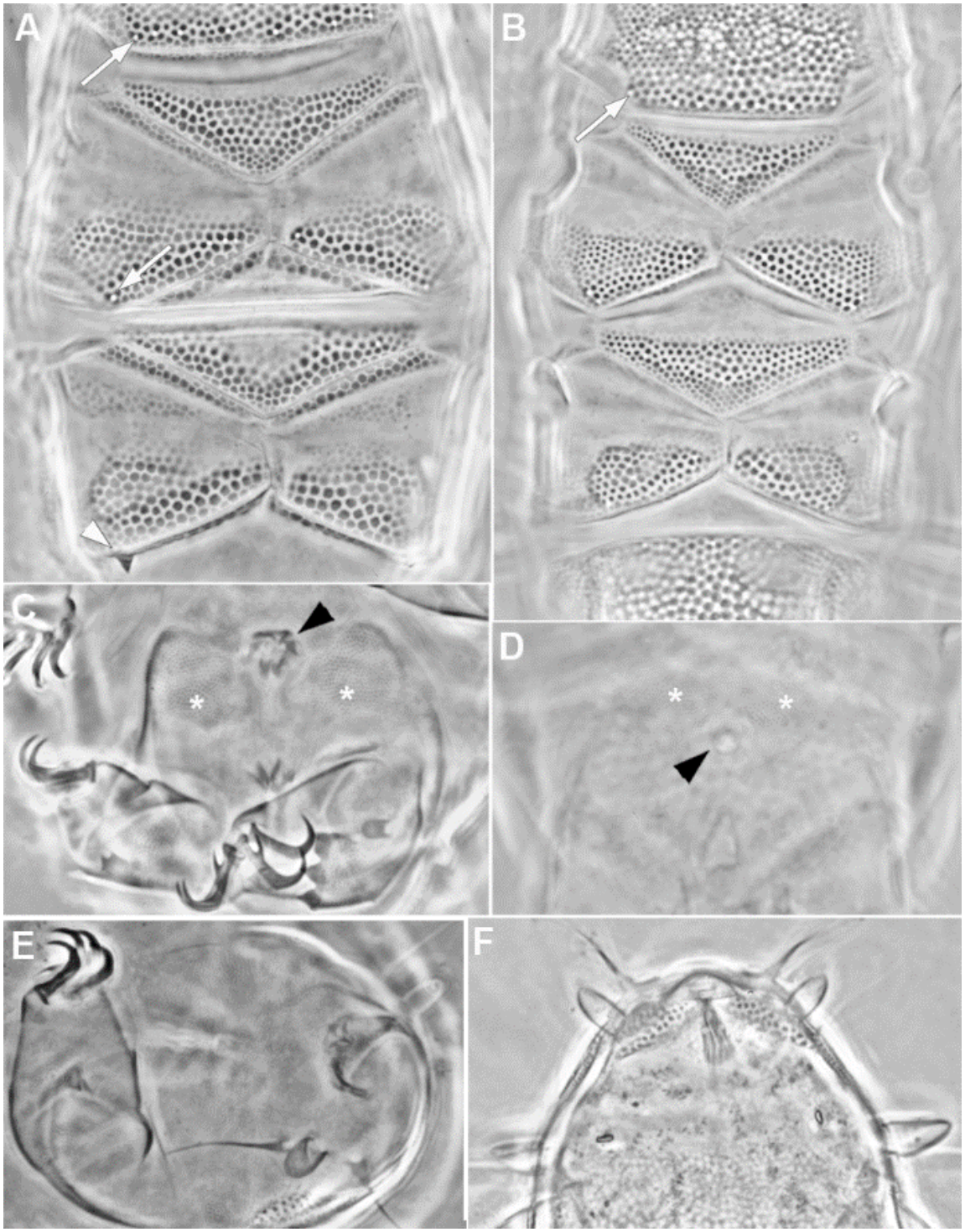


Figure 4. *Diploechiniscus dimorphus* n.sp. female (SEM). A. Dorsal view. B. Ventral view (arrowhead: small spine in E'). C. Ventral surface of the head cuticle with small dots and cephalic plates (asterisks). C. Ventral surface of the head cuticle with small dots and cephalic plates (asterisks). D. Gonopore (arrowhead), genital plates (asterisks). E. Scapular plate with lines of small pores (arrows). F. Legs of the second and third pairs. G. Second paired plates and terminal plates (arrow: small hole; black arrowhead: small D^d spine; white arrowhead: small spine in E). Scale bars: A-B = 20 µm, C-F = 10 µm.

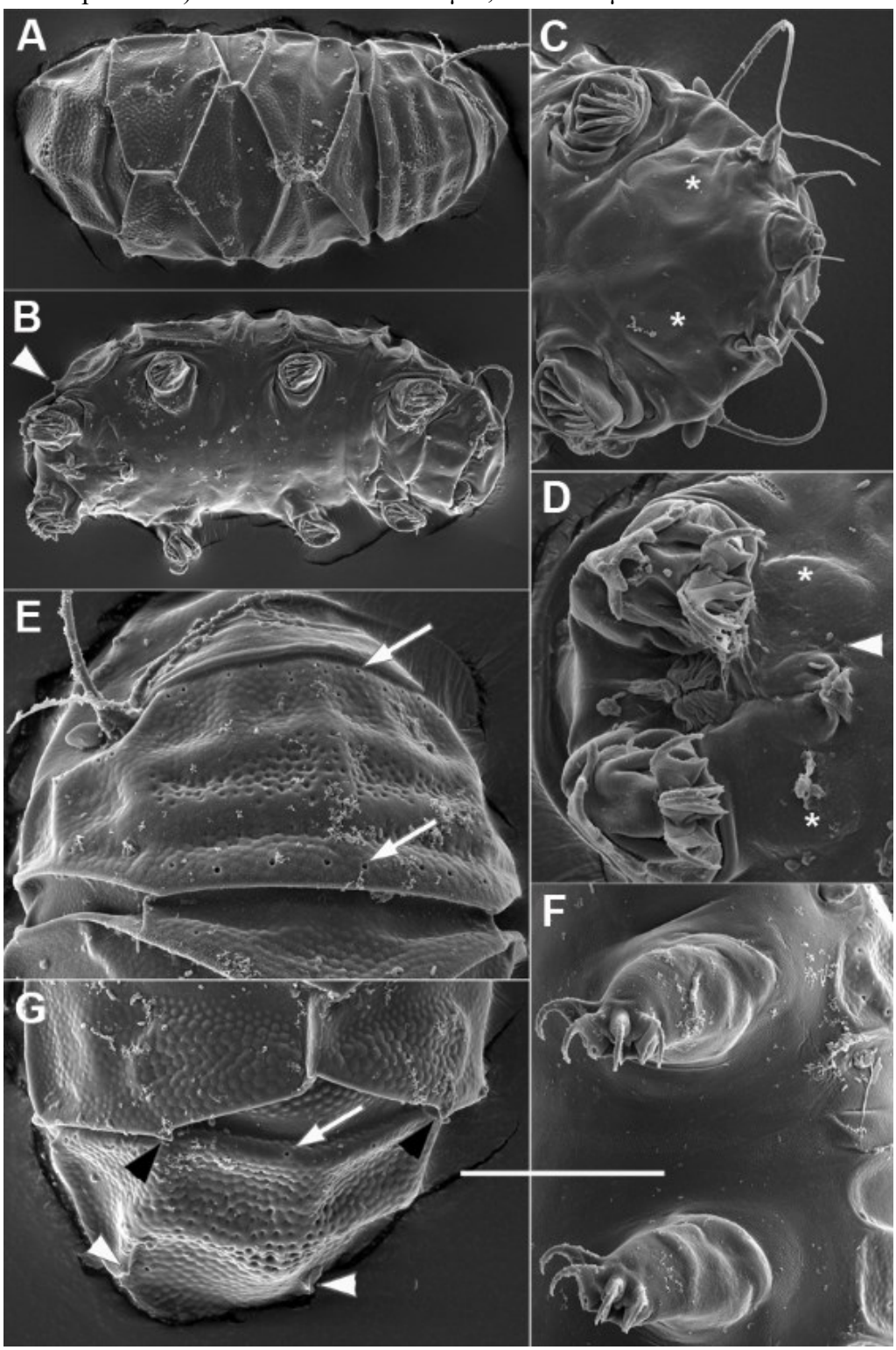
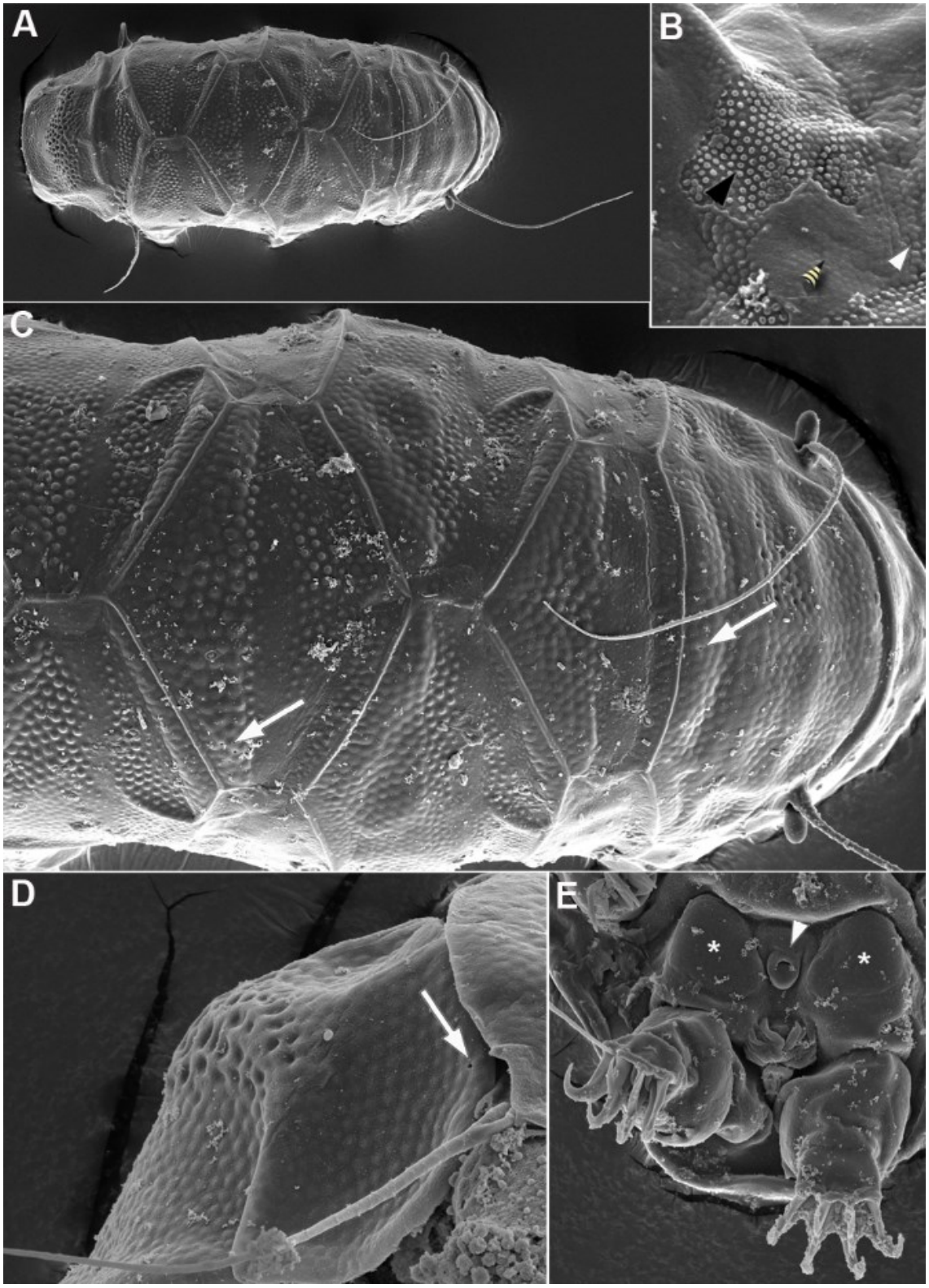


Figure 5. *Diploechiniscus dimorphus* n.sp. male (SEM). A. Dorsal view. B. Ventral cuticle (black arrowhead: cuticular pillars; white arrowhead: granules; striped arrowhead: granules with depressions). C. Dorsal cuticle (arrows: small holes). Terminal plate (arrow: small hole). E. Gonopore (arrowhead), genital plates (asterisks). Scale bars: A = 50 µm, B = 5 µm, C-E = 10 µm.



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Supplementary material

cjz-2024-0040suppla. Raw measurements of *Diploechiniscus dimorphus* n. sp. type series from S1911

cjz-2024-0040supplb. Raw measurements of *Diploechiniscus* spp. from S1911

cjz-2024-0040supplc. MrBayes input file with alignment and partition models

cjz-2024-0040suppld. Complete ML phylogenetic tree

cjz-2024-0040supple. Complete BI phylogenetic tree

cjz-2024-0040supplf. ASAP results

cjz-2024-0040supplg. bPTP results

cjz-2024-0040supplh. COI p-distances table