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A New Species of Bisexual *Milnesium* (Eutardigrada: Apochela) Having Aberrant Claws From Innhovde, Dronning Maud Land, East Antarctica

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There have been several records in the last 60 years for East Antarctica for *Milnesium tardigradum* Doyère, 1840 sensu lato, now considered a species complex. During the 56th Japanese Antarctic Research Expedition summer operation (2014–2015), a new tardigrade species in the genus *Milnesium* Doyère, 1840 was found in an ice-free Innhovde area along Lützow-Holm Bay, Dronning Maud Land, East Antarctica. The new species has aberrant claws with four to seven points on each secondary claw branch, which distinguishes it from other *Milnesium* species. A male specimen was found in the population and evidence showed that an isolated adult female moulted twice without oviposition. This strongly suggested bisexual reproduction for this population. The new species, *Milnesium rastrum* sp. nov., is described with its phylogenetic position and a discussion on the reproductive strategies for the harsh environments.

Key words: Lützow-Holm Bay, tardigrade, bisexual reproduction, *Milnesium rastrum*, *Milnesium tardigradum*, Syowa-Station

INTRODUCTION

The genus *Milnesium* Doyère, 1840 was established with the description of *Milnesium tardigradum* Doyère, 1840 from Saint-Maur near Paris. This tardigrade was characterised by four claws on each leg, with two long primary claws and two short secondary claws, with the anterior secondary claw divided into three branches and the posterior with two branches (Doyère, 1840). The second species, *Milnesium alpigenum* Ehrenberg, 1853, was distinguished by having three branches on all secondary claws (Ehrenberg, 1853; illustrated in Ehrenberg, 1854). Despite the differing number of branches (or points) on the secondary claws, this character was regarded as merely intraspecific variation by early researchers (Richters, 1902, 1904, 1908; Marcus, 1936). Later, Michalczyk et al. (2012) emphasized the taxonomic importance of the secondary claws and proposed a notation system to describe the claw configuration (CC). Among more than 40 described species in this genus, almost all species have two or three branches (reviewed by Suzuki, 2022) but *M. quadrifidum* Nederström, 1919 has four branches on each secondary claw. A more recent exception, *M. wrightae* Kaczmarek et al., 2019, has a very small fourth point near the base of the secondary claws of leg IV. The significant exceptions in publications were of specimens from East Antarctica, which were identified as *M. tardigradum* in spite of having five or more branches on the secondary claws (Sudzuki, 1964; Dastych, 1984).

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During the 56th Japanese Antarctic Research Expedition (JARE-56) summer operation (2014–2015), field investigation and monitoring of terrestrial ecosystems were carried out by three of the authors (ACS, MT, and RN) in several areas along Lützow-Holm Bay, Dronning Maud Land, East Antarctica. The main aim of the investigation was to examine and characterize diversity and distribution of the meiofauna and the microbiome in the ice-free areas in this region. In this context, re-examination of *Milnesium* sp. mentioned above was planned, and microscopic examinations were performed as much as possible during the short field stay. Although *Milnesium* specimens described by Sudzuki (1964) were collected from the Langhovde area (69°13′S, 39°45′E) during JARE-5 (1960–1962), this time we found this tardigrade during the first biological survey of the Innhovde (69°51′S, 37°06′E) region.

MATERIALS AND METHODS

Sample collection, rearing conditions, and microscopy

The ice-free area of Innhovde is situated at ca. 120 km southwest of Syowa Station (Figs. 1, 2). Along the north-eastern slope near the coast are exposed rocks, about 3 km long from the NW end (ca. 10 m above sea level) toward the SE end (160 m a.s.l.). We conducted a field study in the Innhovde area on 11 January 2015. Limited vegetation occurred in the northern quarter of the area; in contrast, the rest of the southern area was relatively dry with little vegetation, and lakes were still frozen at the time of our visit. Mosses and lichens from the northern area were collected in paper bags and kept dried in cold storage, at 4°C during the expedition, then later at −20°C in a cold room at National Institute of Polar Research (NIPR), Tokyo.

Several samples were examined during the expedition in our field laboratory at Kizahashi-hama, Skarvsnes (69°28′22″S, 39°36′06″E). The samples were divided into several portions, one of which was immediately soaked in Milli Q water and the small animals that were liberated were picked out under a stereo microscope (Wild M3C, Leica). Two adult females and a juvenile of the *Milnesium* species were found in the moss *Ceratodon purpureus*. The adult specimens were mounted on glass slides in gum-chloral solution, and the juvenile was fixed and stored in ethanol for DNA analysis. Images of the slide specimens were taken at the field laboratory with a digital camera (NEX5-N, Sony) on a compound microscope (Nikon L-Ke) with differential interference contrast (DIC), and again later using another microscope (BX50, Olympus) with DIC. The rest of the samples were returned to Japan, where the remainder of the moss sample that had yielded the *Milnesium* specimens at the field laboratory was examined. Additional specimens comprising an exuvia with three eggs, an adult female, a premature simplex individual, and a moulting male were extracted. The premature simplex specimen was fixed with ethanol for DNA analysis, while the moulting male and three hatchlings from the eggs were mounted on microslides. The adult female, which was presumed to be the mother of the three hatchlings, was reared in a plastic dish and the life history was observed under a stereo microscope (Wild/Leica M-10). The rearing conditions were as described in Suzuki (2003) with a slight modification, using rotifers *Lecane inermis* (Bryce, 1892) as a food source, and at 4 or 10°C under 16L:8D photoperiod. After this female individual died, the specimen was processed for scanning electron microscopy (SEM). It was fixed in 10% formalin, post-fixed in 1% OsO4 in 0.1 M sodium cacodylate, pH 7.2 for 1 hr at room temperature, dehydrated through a

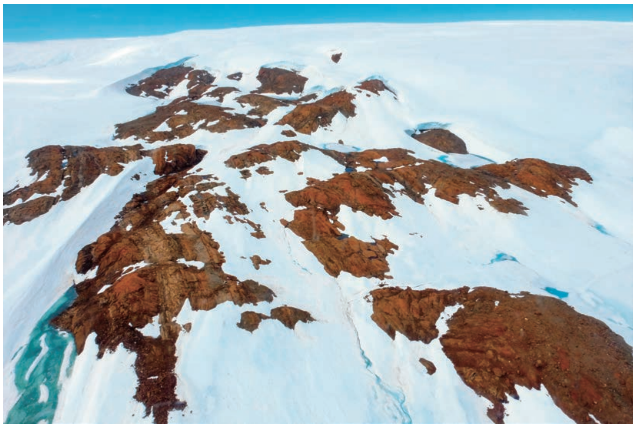

Fig. 1. Aerial view of Innhovde on 11 January 2015, from NW to SE direction.

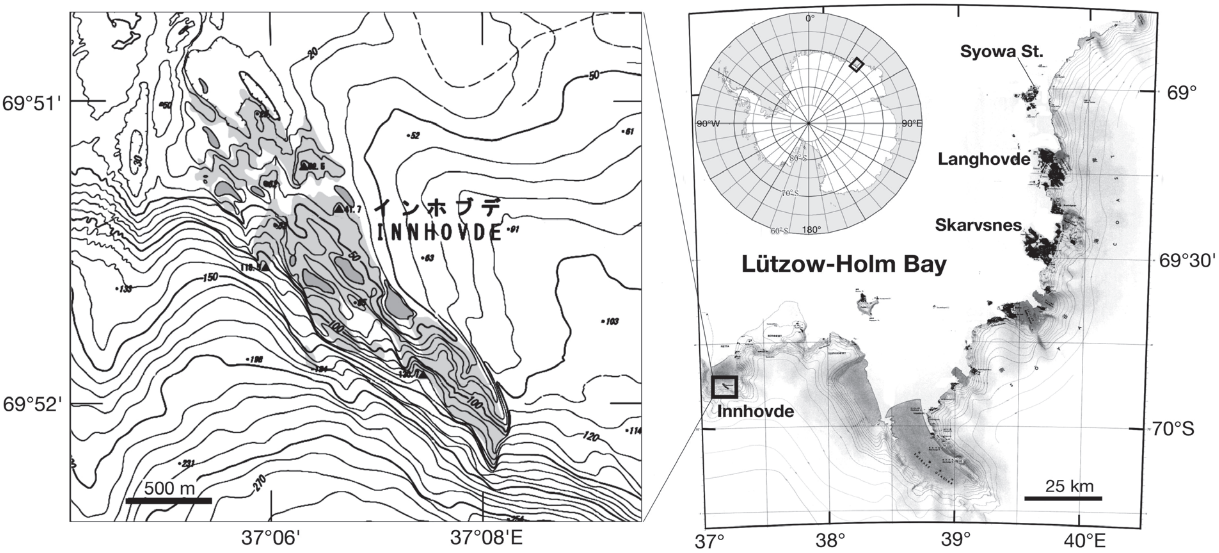

Fig. 2. Maps showing Innhovde. Based on maps by Geospatial Information Authority of Japan (https://www.gsi.go.jp/antarctic/02.html) and modified.

graded series of ethanol and 100% t-butyl alcohol, frozen at 4°C overnight, and lyophilized using a JFD-320 (JEOL). The specimen was mounted on an aluminium stub, sputter-coated with gold, and observed with a JSM 6510 (JEOL) at 20 kV.

Specimens for morphometry were observed under an Olympus BX-50 DIC microscope with a digital camera, and measurements were taken using ImageJ (https://imagej.nih.gov/ij/). Structures were measured when their orientation was suitable. Body length was measured from the anterior extremity to the end of the body excluding the hind legs. Buccal tube length and stylet support insertion point were measured according to Tumanov (2006). Buccal tube width (diameter) was measured at the anterior, standard, and posterior positions (Michalczyk et al., 2012). The *pt* index (Pilato, 1981) was used for comparison, i.e., the percentage of the length of each structure relative to the buccal tube length, expressed in italics. Illustrations were made using a drawing tube attached to a BX-50 microscope and finished with Inkscape software (https://inkscape.org/). The terminology for the *Milnesium* claw system generally followed Camarda et al. (2022), i.e., we now replace the terms “primary/secondary branches” in the previous works (reviewed in Suzuki, 2022) by “primary/secondary claws”, and instead we call the hooks, or points, on the secondary claws “branches”. Following Suzuki (2022), we avoid using the directional terms *internal*/*external* for leg I–III, and describe the CC by the revised notation system (Suzuki, 2022) with a modification, i.e., the number of “branches” on the “secondary claws” expressed in brackets as {*anterior*/*posterior*} in order through all legs. The position of legs was indicated by a superscript Roman numeral behind the brackets, if necessary. By this notation, the adult CC of *M. tardigradum* is expressed simply as {3-2}, or more precisely $\{3\text{-}2\}^{\mathrm{I–IV}}$. Specimens of the new species in the present study have a different number of branches, even on the left/right legs in one individual, so that every CC was described for each leg.

Other samples of vegetation collected from Antarctica during JARE-56 have been kept at NIPR for future investigation.

DNA extraction and sequencing

DNA extraction was performed as described by Kagoshima et al. (2013). Each specimen was transferred individually to 20 μL of 0.25 N NaOH in a 0.2 mL tube and kept at room temperature for 12 hr. The tube was heated for 3 min at 95°C, and 4 μL of 1 M HCl and 10 μL of 0.5 M Tris-HCl (pH 8.0) were added to neutralize the base followed by 1 μL of 2% Triton X-100. The lysate was heated for a further 3 min at 95°C and stored at −20°C. Polymerase chain reaction (PCR) amplification was performed with primers as follows: for the 18S rRNA gene we used the primers SSU04F and SSU81R (Blaxter et al., 1998), for the 28S rRNA gene the primers were 28S_Eutar_F (Gąsiorek et al., 2017) and 28SR0990 (Mironov et al., 2012), for ITS-2 they were ITS3 and ITS4 (White et al., 1990), and for COI the primers were LCO1490 and HCO2198 (Folmer et al., 1994). PCR conditions for the 18S rRNA gene and COI were: 94°C for 2 min, followed by 40 cycles of 94°C for 10 sec, 52°C for 30 sec, and 72°C for 10 min. Conditions for the 28S rRNA genes and ITS-2 were: 94°C for 2 min, 40 cycles of denaturing at 94°C for 30 sec, annealing at 50°C for 30 sec, extension at 68°C for 75 sec, and final extension for 7 min. Sequencing reactions were performed with Big-Dye terminator cycle sequencing kits and run on the ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) for 18S rRNA gene and COI, and sequencing for the other two regions was performed by Eurofins Genomics (Japan). The sequences were checked and trimmed with SnapGene Viewer, a software from Insightful Science (snapgene.com).

Phylogenetic analyses

For phylogenetic analyses, we used the publicly available *Milnesium* spp. sequences of 18S rRNA, 28S rRNA, ITS-2, and COI in the DDBJ/ENA/GenBank databases as shown in Supplementary Table S1. Antarctic taxa and some species with published sequences were added to the list. The sequences were aligned using MAFFT v7.222 (Katoh et al., 2002; Katoh and Toh, 2008) with the default setting, then obtained alignments were checked by using MEGA7 (Kumar et al., 2016) and trimmed manually to 193 bp in 18S rRNA, 564 bp in 28S rRNA, 104 bp in ITS-2, and 478 bp in COI. The p-distances were calculated with MEGA7.

Phylogenetic analyses were run with COI and 18S + 28S + ITS-2 + COI concatenated sequences. The concatenated sequences included only the taxa with the COI sequence and at least one nuclear DNA sequence. Four Antarctic COI sequences: KJ857001, KJ857002, KP013598, and KP013613, were added for the concatenated sequences even though the taxa with only COI sequence was available. The best-fit evolution model and Maximum Likelihood (ML) topologies were calculated with IQ-TREE v1.6.12 (Minh et al., 2020) by 10,000 rapid bootstrap replicates. TVM+F+G4, GTR+F+I+G4, K3Pu+F+G4 and HKY+F+R7 were estimated to ITS-2, 1st, 2nd and 3rd codon of COI, respectively. In addition, K2P+G4 and T2P+R5 were estimated to the respective partitions for 18S and 28S of the concatenated sequence. Bayesian Inference (BI) probabilities were calculated with MrBayes v3.2.6 (Ronquist and Huelsenbeck, 2003; Ronquist et al., 2012) under GTR+I+G model with “nst = 6 rates = invgamma” option, and the analyses were run for over 5,000,000 generations, sampling the Markov chain every 1,000 generations. Moreover, an average standard deviation of split frequencies of < 0.01 was confirmed. The trees were visualised in FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree).

Genetic species delimitation

Genetic species delimitation was performed at the bPTP server (https://species.h-its.org/ptp) with the following settings: 100,000 MCMC generations, 100 thinning, 0.1 burn- in via Poisson Tree Processes (PTP) model, excluding the outgroups (Zhang et al., 2013).

RESULTS

Taxonomic account

Class **Eutardigrada** Richters, 1926
Order **Apochela** Schuster et al., 1980
(amended by Morek et al., 2020a)
Family **Milnesiidae** Ramazzotti, 1962
(amended by Morek et al., 2020a)
Genus ***Milnesium*** Doyère, 1840
Milnesium rastrum Suzuki, Sugiura, Tsujimoto, and McInnes, sp. nov.
(Figs. 3–10; Tables 1, 2)
Milnesium tardigradum: Sudzuki, 1964, pl. IX, figs. 1–18; Dastych, 1984, fig. 33a–d.

Diagnosis. *Milnesium* with four–seven branches on each secondary claws. Live adult body orange in color under stereomicroscopy. Eyes present. Male present, with modified claw on leg I. The presence of a male in the population suggests sexual reproduction.

Materials examined. *Holotype*: Adult female (slide No. NSMT-Tg-334), collected by the first author on 11 January 2015, mounted in gum-chloral solution. *Type locality*: Innhovde (69°51′13.8″S, 37°06′12.9″E), Dronning Maud Land, East Antarctica. All other materials examined from the same locality. *Paratypes*: A male at the stage of moult between juvenile and adult with newly formed adult claws (NSMT-Tg-335) and three hatchlings (NSMT-Tg-336), all mounted in gum-chloral solution. Deposited in National Museum of Nature and Science, Tsukuba, Japan. Another

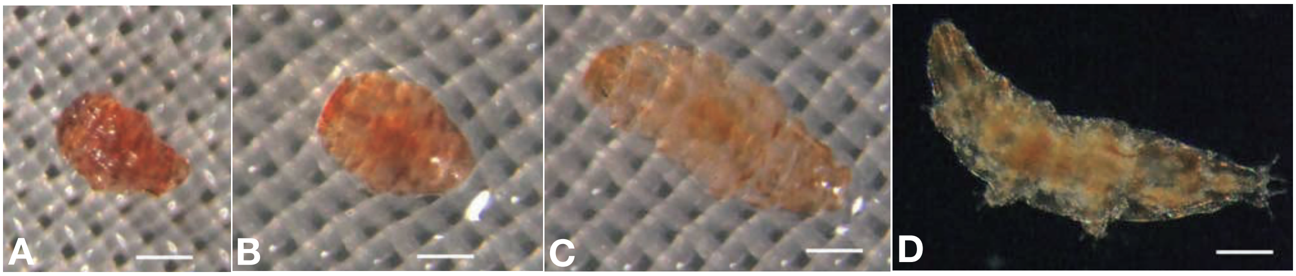

Fig. 3. Image of live specimen of *Milnesium rastrum* sp. nov. under the stereomicroscope. Adult female (Female-3). **(A)** 'Tun' on the nylon mesh. **(B)** Body after 7 minutes of rehydration. **(C)** Expanded body, 15 minutes after rehydration. **(D)** Revived animal walking on agar, 38 minutes after rehydration. Scales, 100 μm.

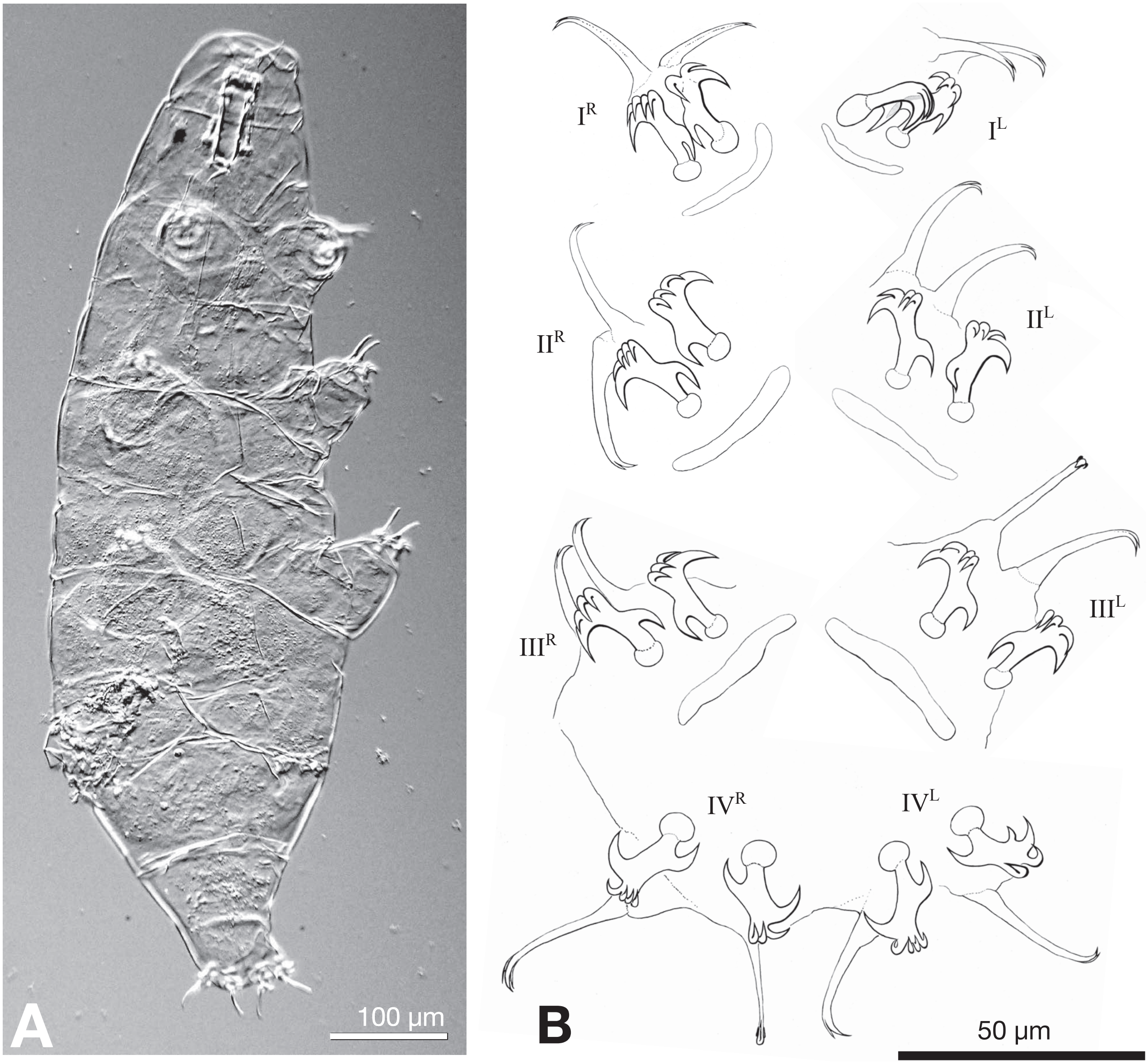

Fig. 4. Adult female of *Milnesium rastrum* sp. nov. **(A)** Holotype habitus. **(B)** Claws on each leg. The Roman numeral indicates the position of the leg, with the superscript showing right or left.

adult female (designated as female-2 in this study) was regrettably lost during an unsuccessful attempt at restoration after the mounting media deteriorated. However, we were left with good photographs of the specimen and measurements shown in this article.

Other materials: An exuviae (NSMT-Tg-337) with three

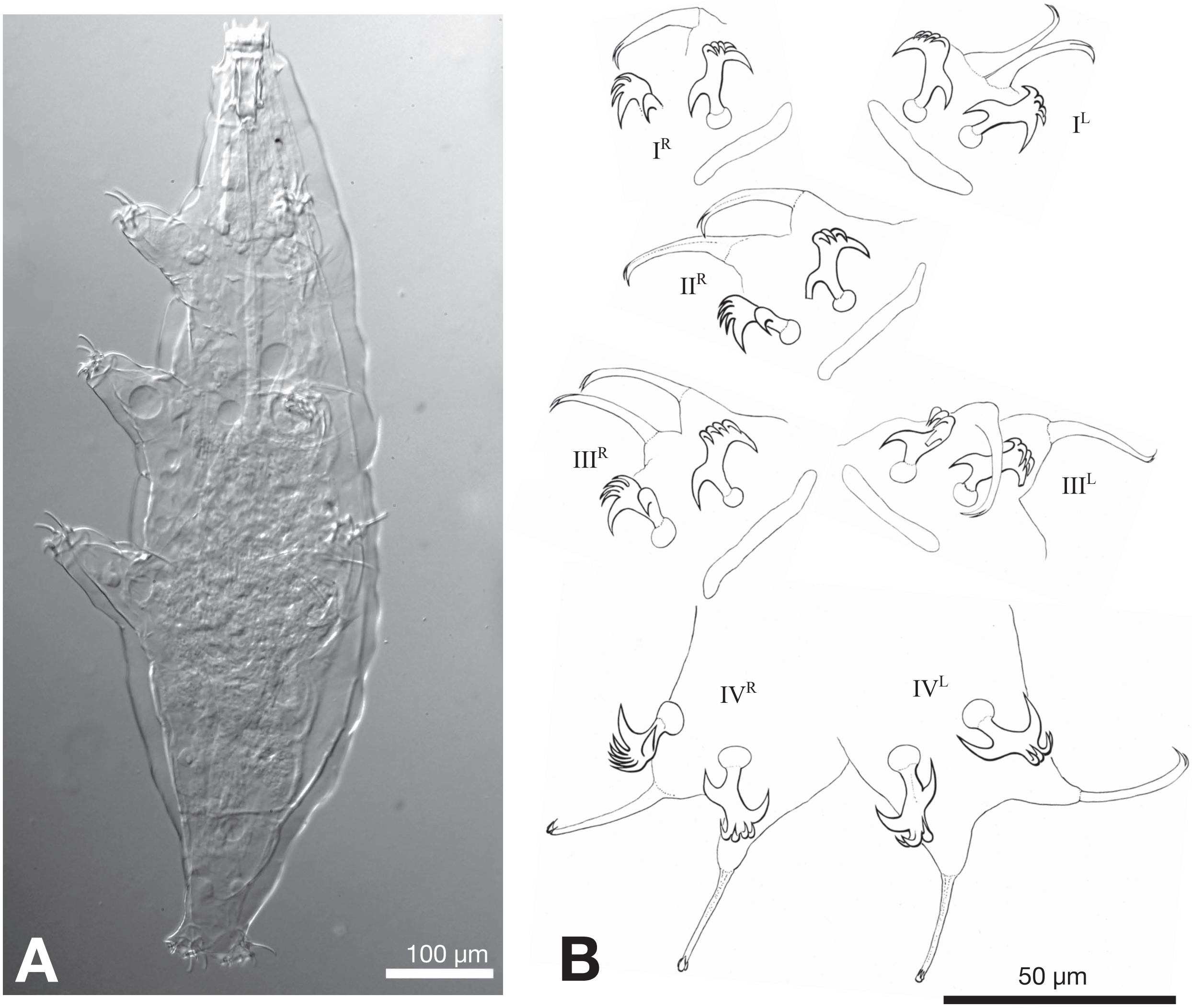

Fig. 5. Adult female of *Milnesium rastrum* sp. nov. **(A)** Female-2 habitus. **(B)** Claws on each leg. The illustration of the leg II^{L} is missing due to its unfavorable position.

egg shells that produced the three paratype hatchlings. An adult female (designated as female-3 in this study) was reared for 2 months, during which time she moulted once without oviposition (exuviae mounted in gum-choral, NSMT-Tg-338), and a second time but died in the process. Female-3 was prepared and mounted on an aluminium stub for SEM (NSMT-Tg-339). Two further specimens were used for DNA analyses.

Etymology. The specific name *rastrum* means a rake in Latin, and especially a 5-pointed writing implement to draw lines for sheet music; representing the shape of the secondary claws with many branches.

Description. All measurements shown in Table 1.

Adult females (Figs. 3–7): Orange color in living specimens (Fig. 3), large (holotype, 815 µm long, Fig. 4), cuticle smooth. Black eyes present. Six peribuccal and two lateral papillae, six peribuccal lamellae. Buccal apparatus of the *Milnesium* type, buccal tube wide and long (Fig. 6A). Stylet supports inserted on the buccal tube at 62–64% of the length. Pharyngeal bulb pear-shaped, without placoids or septum. Primary claws slender, with two well-developed accessory points (Fig. 6B–D). Secondary claws with 4–7 branches (Figs. 4, 5, 6B–D), holotype CC, left {5-5}^{I}{5-5}^{II}{5-5}^{III}{5-6}^{IV}-right {4-6}^{I}{5-6}^{II}{5-5}^{III}{6-5}^{IV}. Female-2 CC, left {6-6}^{I}{?-?}^{II}{5-6}^{III}{6-5}^{IV}-right {6-5}^{I}{5-6}^{II}{6-6}^{III}{7-6}^{IV}. Exuviae of female-3 CC, left {5-4}^{I}{6-5}^{II}{5-5}^{III}{5-5}^{IV}-right {5-4}^{I}{5-6}^{II}{5-5}^{III}{5-5}^{IV}. Exuviae with three egg shells CC, {?-4}^{I}{5-5}^{II}{6-5}^{III}{6-6}^{IV}-right {5-4}^{I}{5-5}^{II}{?-5}^{III}{?-5}^{IV}. Round thickening at the base of each secondary claw. A long cuticular bar under claws I–III.

Moulting male (Fig. 7): Body length, 585 µm; buccal tube length, 36.6 µm; buccal tube standard width, 13.9 µm; stylet support insertion point, 24.2 µm [*pt* = *66.1*]. Newly generated adult seen through the cuticle of the juvenile. The external cuticle has juvenile-type claws on leg I, while the new adult claws exhibited the typical male character with the robust hook with accessory points and basal branch on the secondary claws. Other secondary claws four or five branches: left {male}^{I}{?-?]^{II}{5-5}^{III}{?-?}^{IV}-right {male}^{I}{4-5}^{II}{5-4}^{III}{5-5}^{IV}. Juvenile cuticle CC, left {4-4}^{I}{5-5}^{II}{4-5}^{III}{5-5}^{IV}-right {4-5}^{I}{4-4}^{II}{5-4}^{III}{5-5}^{IV}.

Hatchlings (Fig. 8): Body transparent. Hatchling-1 CC,

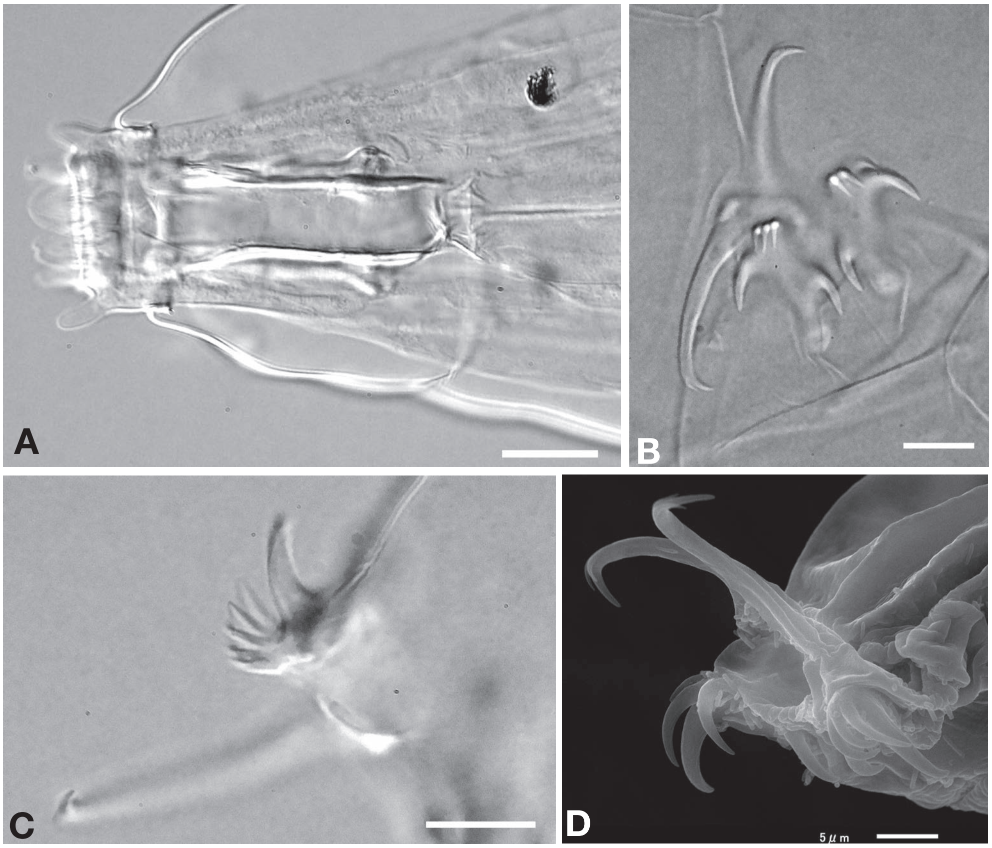

Fig. 6. Female of *Milnesium rastrum* sp. nov. **(A)** Head region showing the buccal apparatus, peribuccal structures and eyes. Female-2. Scale, 20 μm. **(B)** Claws of right leg II of holotype, {5-6}^{IIR} CC. Scale, 10 μm. **(C)** Anterior secondary claws of right leg IV of female-2, the secondary claw with seven branches. Scale, 10 μm. **(D)** Claws of right leg II, female-3, SEM. Scale, 5 μm.

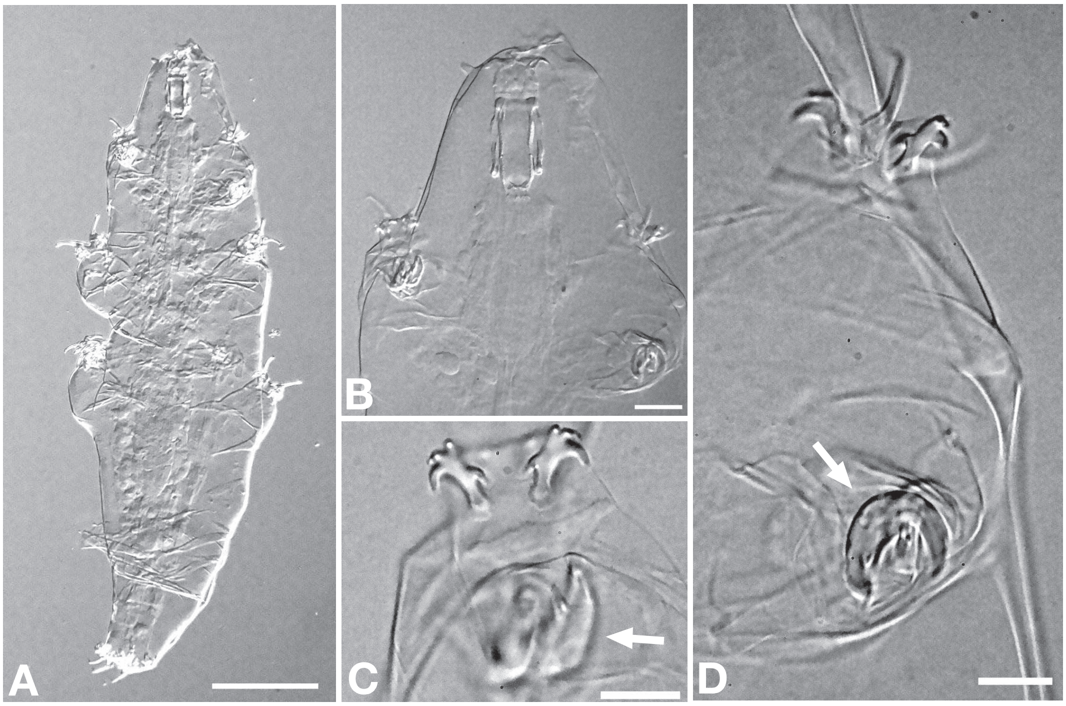

Fig. 7. Moulting male of *Milnesium rastrum* sp. nov. **(A)** Habitus. Scale, 100 μm. **(B)** Anterior half. Scale, 20 μm. **(C)** Right leg I. The juvenile claws at the top, with {5-5} CC. Arrow, the new male claw I under the old cuticle. Scale, 10 μm. **(D)** Left leg I, the juvenile claw (top) and the robust male claw (arrow). Scale, 10 μm.

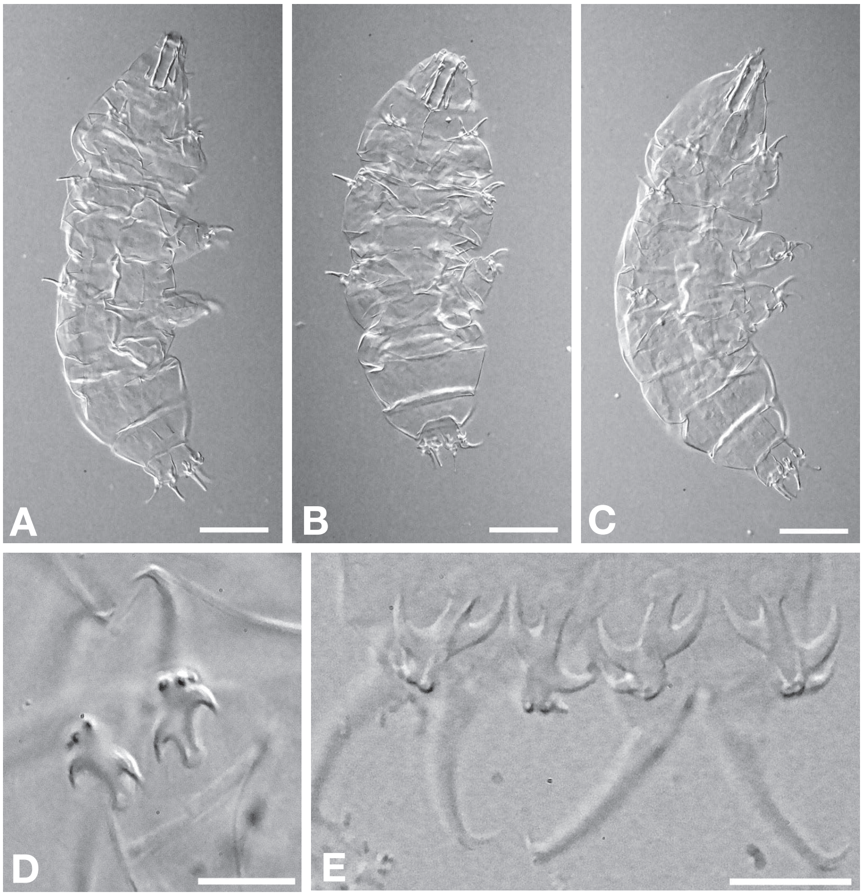

Fig. 8. Hatchlings of *Milnesium rastrum* sp. nov. Habitus of hatchling-1 **(A)**, hatchling-2 **(B)**, and hatchling-3 **(C)**. (**D**) Right leg II of hatchling-1, with {5-5} CC. Scale bar, 10 μm. (**E**) Leg IV of hatchling-3 with {5-5} CC. Scale bar, 10 μm.

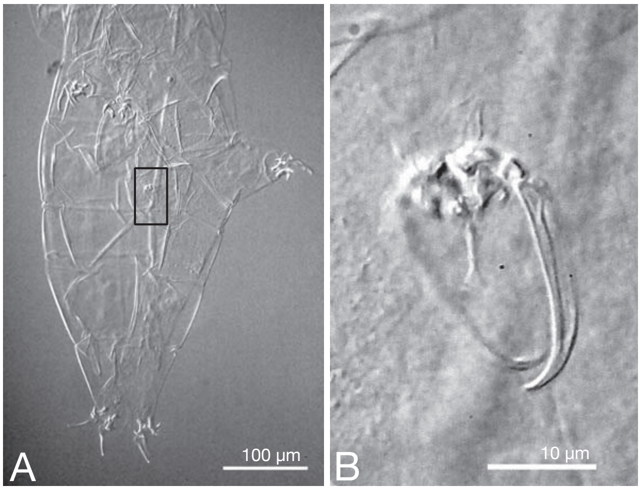

Fig. 9. Exuviae of Female-3. **(A)** Posterior half of the exuviae, including the trophi of rotifer in the hind gut. **(B)** Enlargement showing the trophi.

left $\{4\text{-}4\}^{I}\{5\text{-}4\}^{II}\{4\text{-}4\}^{III}\{5\text{-}4\}^{IV}$-right $\{4\text{-}4\}^{I}\{5\text{-}5\}^{II}\{4\text{-}5\}^{III}\{5\text{-}5\}^{IV}$; hatchling-2 CC, left $\{4\text{-}5\}^{I}\{?\text{-}?\}^{II}\{?\text{-}5\}^{III}\{5\text{-}5\}^{IV}$-right $\{4\text{-}4\}^{I\text{–}IV}$; hatchling-3 CC, left $\{5\text{-}5\}^{I}\{5\text{-}4\}^{II}\{4\text{-}4\}^{III}\{5\text{-}5\}^{IV}$-right $\{5\text{-}5\}^{I}\{4\text{-}4\}^{II}\{5\text{-}4\}^{III}\{5\text{-}5\}^{IV}$.

Observation of a solitary adult female in culture

When the exuviae including three eggs was found, an adult female (female-3) was also retrieved from the same sample. This female, which might have been the mother of the above three eggs in spite of no firm evidence, was temporarily dried and kept in a refrigerator. After a week it was rehydrated (Fig. 3A–D). The dried body was 244 μm long and expanded to 707 μm after rehydration. Once rehydrated it was put into a 35-mm dish filled with water and provided with rotifers as the food source. Table 2 shows the summary of the observations of female-3 for more than 3 months. The movement of the female was very slow, and its eating behavior was not directly observed. However, its gut sometimes looked full of some grey substance.

At day 19 after rehydration, the female lost her buccal apparatus going into simplex (i.e., the moulting stage), but no distinct eggs were discerned in her ovary. It took about 10 days at 10°C to form new buccal apparatus (day 29). Two days later (day 31), the locomotive activity ceased, and apolysis, i.e., detachment of the old cuticle from the newly formed epidermis, was observed. It took another week to finish the ecdysis (day 38). The remains of a rotifer trophi in the exuviae between the 3rd-leg pair (in the old cuticle of the hind gut) provided evidence that this female had been eating the rotifers (Fig. 9).

The new-instar female continued her slow locomotion

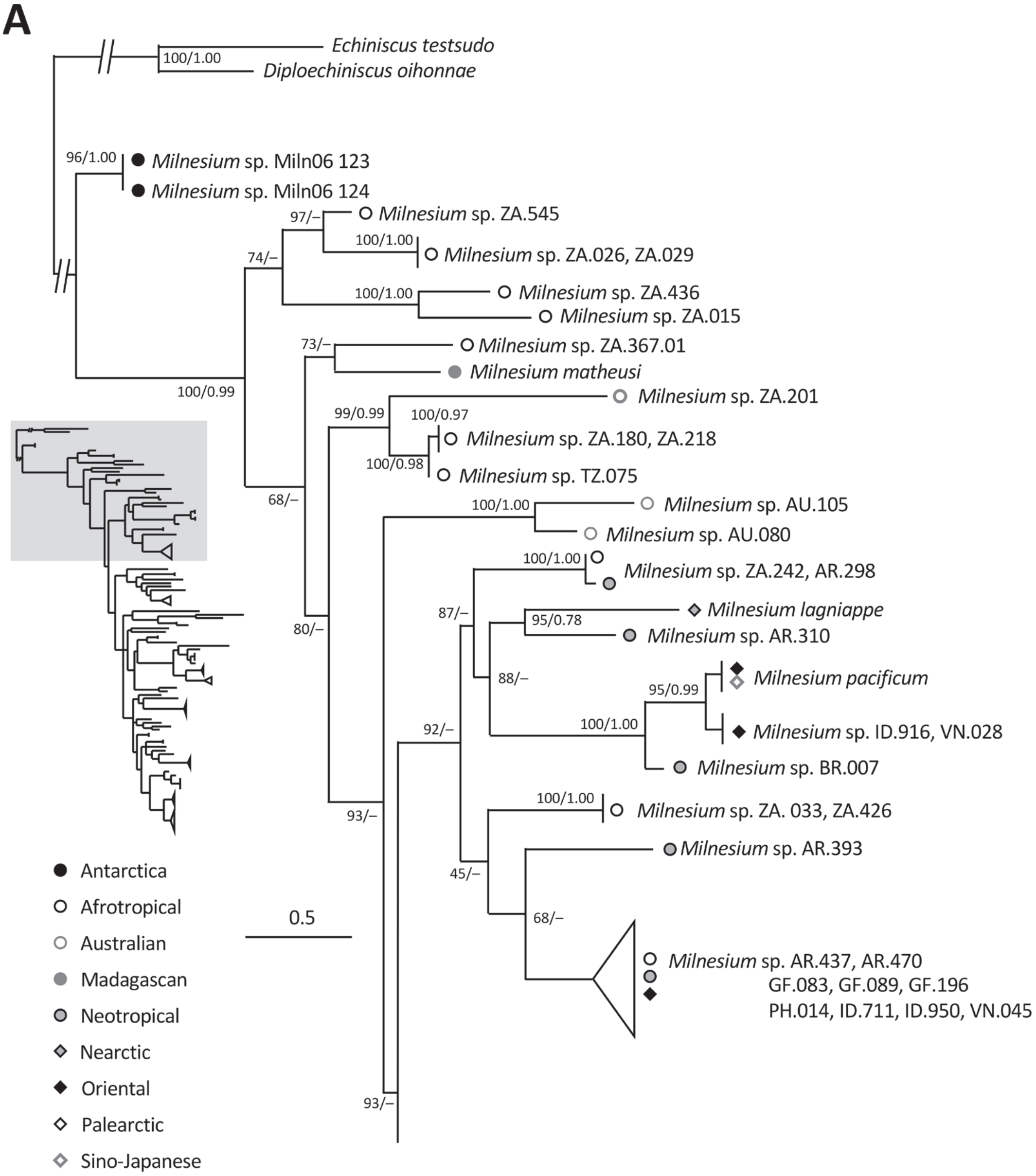

Fig. 10. Continued.

for a month until day 68, and then the next moulting began, indicated by the loss of the buccal tube again. The ovary, as before, had no large oocytes. It took 14 days to recognize the beginning of the buccal tube regeneration. At day 86, the observation was interrupted for 10 days at 4°C (caused by a conference trip of the first author). By day 96, when the animal was returned to 10°C, the new buccal tube had already formed. However, she did not complete ecdysis and apparently died at day 103. The body was fixed and processed for SEM observation (Fig. 6D). Overall during this observation, this female moulted twice without egg laying.

Genetic species delimitation

The DNA sequences of *Milnesium rastrum* sp. nov. were deposited in the DDBJ with the following accession numbers: LC721285 (18S rRNA), LC721286 (28S rRNA), LC721288 (ITS-2), and LC721287 (COI). A summary of genetic p-distances is shown as follows:

18S rRNA: 0.0%–1.9%, with the most similar species being *Milnesium* sp. CJS-2007a MilnC 010 (EF632492), *Milnesium* sp. CJS-2007a MilnC 025 (EF632493), *Milnesium* sp. Miln06 108 (EU266922), and *Milnesium* sp. Miln05 141 (EU266923) from Antarctica, and the least similar being *M. dornensis* Ciobanu et al., 2015 RO.008 (MK484071) from Romania.

28S rRNA: 5.1–9.5%, with the most similar species being *Milnesium* sp. UG.006 from Uganda (MK484006), and the least being *Milnesium* sp. AU.080 from Australia (MK483992).

ITS-2: 15.3–44.4%, with the most similar species being *Milnesium* spp. including PH.014 from the Philippines (MK484029), AR.470 from Argentina (MW538052), GF.196 from French Guiana (MW538055), ID.432, ID.711, ID.940 and ID.950 from Indonesia (MW538057, MW538058,

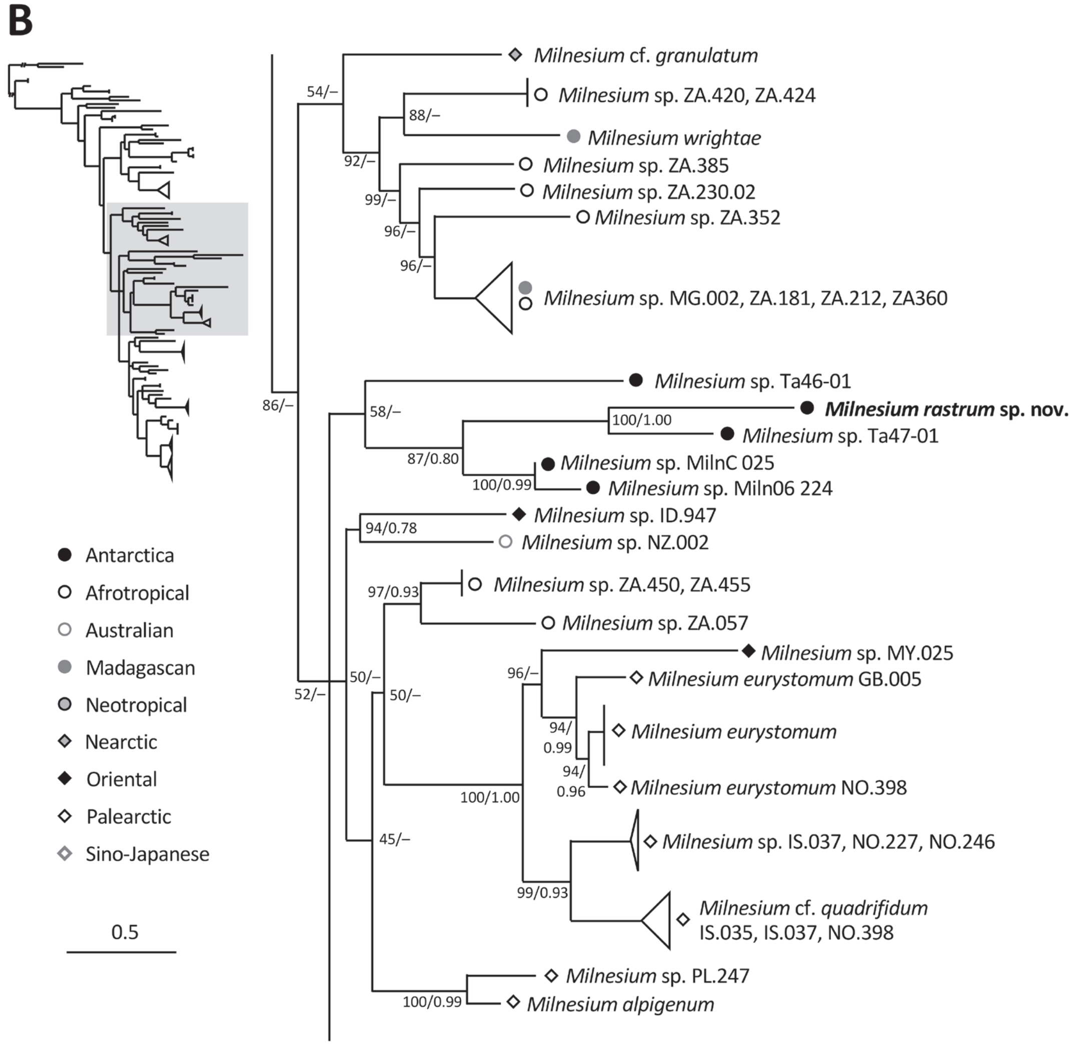

Fig. 10. Continued.

MW538060 and MW538062, respectively); and the least similar species being *Milnesium* sp. ZA.087 from South Africa (MW538083).

COI: 18.0–32.2%, with the most similar species being *Milnesium* sp. Ta47-01 from Antarctica (KJ857002), and with the least similar species being *M. berladnicorum* Ciobanu et al., 2014 ZA.040 from South Africa (MW560657).

Phylogeny

The phylogenetic trees based on the COI and concatenated sequences are shown in Fig. 10 and Supplementary Figure S1, respectively; the supported values of BI trees were usually low, thus the phylogenetic trees based on the ML trees are shown. The COI ML tree indicates that *M. rastrum* sp. nov. is a separate species with 100% probability by both maximum likelihood and Bayesian supported solutions, and the new species is included in a clade with Antarctic specimens (*Milnesium* spp. Miln06 224, MilnC 025, Ta47-01, and Ta46-01).

DISCUSSION

Differential diagnosis

Milnesium rastrum sp. nov. is clearly distinguished from all other congeners by having four–seven branches on the secondary claws, which CC is probably expressed as {(4–7)-(4–7)}. *Milnesium quadrifidum* has {4-4} CC, *M. wrightae* has $\{3\text{-}3\}\{4\text{-}4\}^{IV}$ with a minute 4th branch near the base of the secondary claw, and all other reported species have either CC of {3-3}, {3-2}, or {2-2}, or a mixture of these combinations (Suzuki, 2022).

Two species have already been described from Antarctica: *Milnesium antarcticum* Tumanov, 2006 from King George Island and *M. validum* Pilato et al., 2017 from Victoria Land, both have {3-3} CC. In addition to the CC difference, *M. rastrum* sp. nov. also differs from:

Milnesium antarcticum by having a shorter buccal tube for a similar body length (range: 62.5–64.1 µm in the new species and 67.0–74.7 µm in *M. antarcticum*); the adult body length for *M. antarcticum* was reported to exceed 800 µm (Tumanov, 2006) and thus may be similar to that of the new species; and by having a more cephalic position of stylet

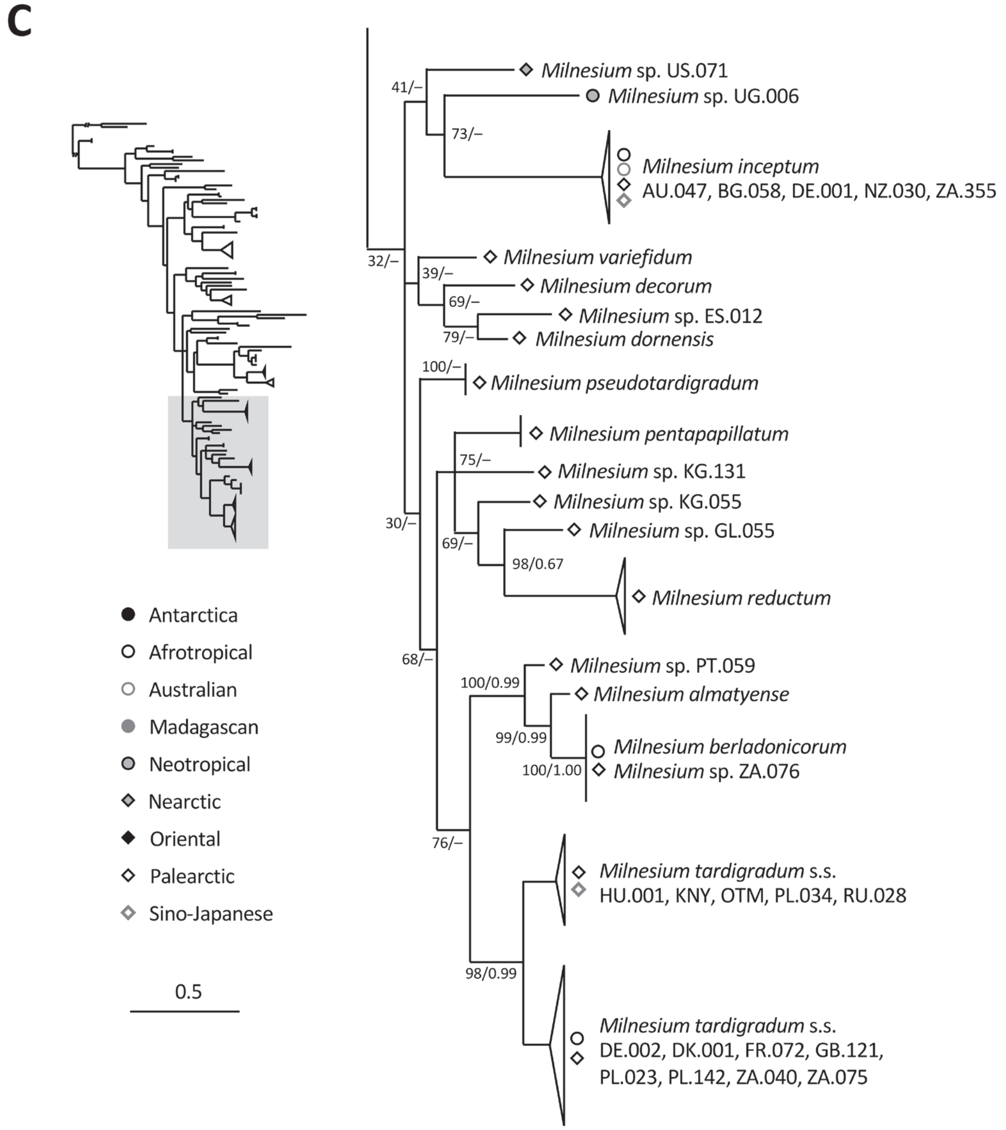

Fig. 10. Maximum likelihood phylogenetic trees based on COI. **(A–C)** indicate the expanded tree corresponding to the legend. The number on each node indicates bootstrap value (only above 90) and Bayesian posterior probability (only above 0.9). Symbols indicate the distribution of each taxon explained in the legend. N.B. *M. pacificum* was not attributed clearly to a specific locality, requiring two symbols. Scale bar indicates substitutions per position.

support insertion point (range: [62.0–64.2] in the new species and [70.0–73.7] in *M. antarcticum*).

Milnesium validum, by having a shorter buccal tube with respect to the body length (percent ratio 6.9–7.9 in the new species and 10.4–11.5 in *M. validum*).

Other records of Antarctic *Milnesium* specimens

The distribution of *Milnesium* spp. recorded so far in and around Antarctica is shown in Fig. 11. The morphological trait of multiple branches observed in the new species from Innhovde has already been reported from other areas within the East Antarctic region. The specimens from Langhovde (Sudzuki, 1964) showed two–five branches on the secondary claws. Although the quality of the illustrations was far from perfect, this description suggested that the samples possibly included specimens with up to five-branched secondary claws. Moreover, although this report did not mention any males, one of the illustrations clearly showed the modified secondary claw of a male (see fig. 12 in Sudzuki, 1964). This indicates that a dioecious *Milnesium* population also occurred at Langhovde, and it is possible these belong to the new species. Co-localization of other *Milnesium* sp. with {3-3} CC has been suggested by the literature that described *M. tardigradum* sensu lato from Molodeznaya, in the vicinity of Lützow-Holm Bay (Utsugi and Ohyama, 1991).

Dastych (1984) compared specimens from King George Island and Evening Mountain (67°39′S, 46°06′E), near Molodeznaya Station, both recorded as *Milnesium tardigradum* sensu lato, describing them as follows: “Specimens from King George Island are typical [...], those from Antarctic Continent belong to the form [...] having variable middle branch of claw. That branch is single in typical specimens […], in mentioned form it is divided into 2–5 teeth, mostly 3–4; […]. A number of teeth is different within this species, often even on one leg.”

Table 1. Measurements of *Milnesium rastrum* sp. nov.

CHARACTER	Adult FEMALE				HATCHLING					
	Holotype		Female-2		Hatch-1		Hatch-2		Hatch-3	
	µm	*pt*	µm	*pt*	µm	*pt*	µm	*pt*	µm	*pt*
Body length	815	*1271*	900	*1440*	338	*1073*	291	*898*	336	*1003*
Peribuccal papillae length	9.1	*14.2*	8.2	*13.1*	4.0	*12.7*	3.9	*12.0*	4.2	*12.5*
Lateral papillae length	7.8	*12.2*	7.8	*12.5*	5.5	*17.5*	4.6	*14.2*	5.5	*16.4*
Buccal tube										
Length	64.1		62.5		31.5		32.4		33.5	
Stylet support insertion point	39.7	*62.0*	40.1	*64.2*	22.2	*70.5*	20.9	*64.5*	21.4	*63.9*
Anterior width	28.6	*44.7*	25.6	*41.0*	12.2	*38.7*	11.6	*35.8*	12.4	*37.0*
Standard width	22.1	*34.5*	18.3	*29.3*	11.2	*35.6*	10.8	*33.3*	11.1	*33.1*
Posterior width	21.4	*33.4*	18.3	*29.3*	10.3	*32.7*	10.5	*32.4*	10.4	*31.0*
Claw 1										
External primary branch	22.9	*35.8*	21.7	*34.7*	11.8	*37.5*	12.3	*38.0*	12.7	*37.9*
External base + secondary branch	16.5	*25.8*	16.4	*26.2*	7.8	*24.8*	8.1	*25.0*	7.7	*23.0*
External spur	7.5	*11.7*	8.8	*14.1*	4.9	*15.6*	4.2	*13.0*	4.6	*13.7*
Internal primary branch	21.1	*33.0*	22.2	*35.5*	10.1	*32.1*	11.9	*36.7*	12.3	*36.7*
Internal base + secondary branch	16.9	*26.4*	16.1	*25.8*	7.0	*22.2*	7.6	*23.5*	7.3	*21.8*
Internal spur	8.5	*13.3*	6.8	*10.9*	4.1	*13.0*	4.2	*13.0*	5.0	*14.9*
Claw 2										
External primary branch	24.0	*37.5*	22.9	*36.6*	12.2	*38.7*	13.7	*42.3*	13.4	*40.0*
External base + secondary branch	17.3	*27.0*	15.1	*24.2*	8.8	*27.9*	7.7	*23.8*	7.4	*22.1*
External spur	8.3	*13.0*	?		4.9	*15.6*	4.6	*14.2*	4.5	*13.4*
Internal primary branch	23.2	*36.3*	22.5	*36.0*	12.6	*40.0*	11.8	*36.4*	11.6	*34.6*
Internal base + secondary branch	16.2	*25.3*	14.7	*23.5*	8.3	*26.3*	7.0	*21.6*	7.9	*23.6*
Internal spur	7.9	*12.3*	?		5.5	*17.5*	4.7	*14.5*	4.8	*14.3*
Claw 3										
External primary branch	24.6	*38.4*	24.1	*38.6*	12.2	*38.7*	13.4	*41.4*	11.3	*33.7*
External base + secondary branch	17.3	*27.0*	14.7	*23.5*	8.7	*27.6*	7.7	*23.8*	8.3	*24.8*
External spur	9.0	*14.1*	6.3	*10.1*	5.5	*17.5*	4.7	*14.5*	5.0	*14.9*
Internal primary branch	23.1	*36.1*	24.0	*38.4*	14.3	*45.4*	12.6	*38.9*	10.8	*32.2*
Internal base + secondary branch	16.1	*25.2*	15.3	*24.5*	7.5	*23.8*	7.6	*23.5*	8.1	*24.2*
Internal spur	8.9	*13.9*	8.5	*13.6*	5.2	*16.5*	4.2	*13.0*	5.3	*15.8*
Claw 4										
Anterior primary branch	26.9	*42.0*	25.8	*41.3*	13.1	*41.6*	13.3	*41.0*	14.5	*43.3*
Anterior base + secondary branch	16.1	*25.2*	16.2	*25.9*	9.1	*28.9*	8.3	*25.6*	8.4	*25.1*
Anterior spur	7.8	*12.2*	11.0	*17.6*	5.3	*16.8*	5.0	*15.4*	6.2	*18.5*
Posterior primary branch	26.1	*40.8*	25.0	*40.0*	13.7	*43.5*	13.9	*42.9*	14.6	*43.6*
Posterior base + secondary branch	17.2	*26.9*	17.1	*27.4*	8.8	*27.9*	8.7	*26.9*	8.4	*25.1*
Posterior spur	7.9	*12.3*	10.2	*16.3*	6.3	*20.0*	5.6	*17.3*	6.5	*19.4*

Dastych's (1984) description of the 'typical' three-branched specimens from King George Island would correspond with the more recent description of *M. antarcticum*, also from King George Island (Tumanov, 2006). The Continental Antarctic 'form' that Dastych (1984) described with four–seven branches on the secondary claws, mostly five–six branches, would match our description for *M. rastrum* sp. nov.

A 'form' of '*Milnesium tardigradum*' was also described from two nunataks, Baileyranten (73°33.9′S, 14°33.8′W, 1141 m a.s.l.) and Engenhovet (74°34′S, 11°01′W, 1757 m a.s.l.) in Heimefrontfjella, Dronning Maud Land (Sohlenius et al., 1996). The paper stated, "*Milnesium tardigradum* is the form already reported from Antarctica (H. Dastych, personal communication)." Although this statement implied a 'form' (Dastych, 1984), it is not clear as to which 'form' the authors were referring.

The COI sequence of *Milnesium* sp. Ta46-01 was obtained from a specimen collected from the Stornes Penisula, Larsemann Hills (Velasco-Castrillón et al., 2015). Our unpublished data indicates there is a population of *Milnesium* in the Larsemann Hills with multiple secondary claw branches.

Table 2. Life history of an adult female of *Milnesium rastrum* sp. nov. (female-3).

Days after rehydration	Observations	Temperature (°C)
0	**Rehydrated**, revived within 15 min. Walking, no sign of interest in rotifers.	15
1	10°C. Slow walking, without preying on rotifers.	10
2	Ditto	10
8	The gut looks full of something.	10
19	**Simplex** beginning, no buccul apparatus.	10
20	Simplex day 2, still walking, immature ovary.	10
21	Simplex day 3, still walking, BT not visible yet.	10
23	Simplex day 5, BT not visible yet.	10
26	Simplex day 8, BT beginning to form.	10
29	Simplex day 11, BT almost complete, still walking.	10
30	Simplex day 12, curling body at the edge of dish.	10
31	Simplex day 13, extended without motion, apolysis.	10
34	Slow movement in the old cuticle.	10
37	Active movement in the old cuticle.	10
38	**New instar** shed old skin. (Simplex day 20)	10
40	Slow walking, gut mostly full with unidentified materials.	10
43	Gut empty, slight sign of interest in rotifer.	10
68	**Simplex**, no buccul apparatus. (New instar day 30) Ovary immature.	10
82	BT beginning to form.	10
86	BT still incomplete, temperature changed	4
96	BT complete, temperature resumed to 10°C	10
100	Curling body, subtle leg movement, not ecdysed yet.	10
103	No movement. Fixed in formalin.	Room temp.

Another study noted the distribution of *Milnesium* sp. from the inland nunataks at the base of the Antarctic Peninsula, Palmer, and Ellsworth Land, and suggested that morphology indicated, “there may be distinct ‘forms’ or speciation occurring at the different sites within the continental, maritime, and sub-Antarctic” (Convery and McInnes, 2005). The details of morphological differences were not stated. However, the COI sequence of *Milnesium* sp. Miln06-224 (in Velasco-Castrillón et al., 2015) was obtained from Ellsworth Land, the region under discussion in Convery and McInnes (2005). There is currently no information about CC of Ta47-01 from Queen Maud Mountain.

Phylogeography

In the ML phylogenetic tree constructed from *Milnesium* COI, *M. rastrum* sp. nov. appeared in a clade with Antarctic strains: *Milnesium* sp. Ta47-01 from the Queen Maud Mountains, *Milnesium* sp., MilnC 025 from Charcot Island, *Milnesium* sp. Miln06 224 from the Ellsworth Mountains, and *Milnesium* sp. Ta46-01 from Stornes Peninsula, Larsemann Hills. The most basal specimens were apparently *Milnesium* sp. Miln06 123 and Miln06 124, both from Marion Island, followed by *Milnesium* spp. from South Africa (ZA.436, ZA.015 and ZA.367.01), *M. matheusi* Kaczmarek et al., 2019 from Madagascar, and *Milnesium* sp. from Australia (AU.080 and AU.105). Afrotropical strains are also found among many other clades in the phylogenetic trees. In comparison, the Nearctic/Palaearctic strains appeared in a more derived position, after the seventh or eighth node (Fig. 10B, C), and are supported by more studies from these regions. This view of the tree accords with the Gondwana origin hypothesis of several lines of tardigrades (McInnes and Pugh, 1998; Guidetti et al., 2017).

It is interesting that Marion Island harbored the most basal *Milnesium* strain. This island is the tip of an active oceanic intraplate volcano, considered to be less than one million years old and with an earliest subaerial eruption estimated by K–Ar dating at ca. 450,000 years ago (McDougall et al., 2001). On the other hand, the split between Parachela and Apochela is estimated to be at 432 million years ago (Mya) (range, 323–540 Mya) during the Palaeozoic, and the most recent common ancestor of *Milnesium* was estimated to appear 162 Mya (range, 116–207 Mya) (Morek et al., 2021), which would coincide with the onset of the Gondwana breakup about 164 Mya (Mueller and Jokat, 2019). Thus, the island is quite young in comparison with *Milnesium* diversification. However, Marion Island is on the Marion Hotspot that has been stationary since 88 Mya and played an active role in the breakup of Madagascar and India (Storey et al., 1995; Torsvik et al., 1998). According to this geographic view, Madagascar would have migrated North, with its *Milnesium* ancestor, away from the Marion Hotspot about 88 Mya. This *Milnesium* population would have evolved in response to various environments, with the extant Madagascan species *M. matheusi* and *M. wrightae*, as the modern representatives. However, this does not explain how *Milnesium* spp. arrived on Marion Island. We cannot say where the ancestral *Milnesium* species existed until we get more spatiotemporal data and hopefully some fossil records. Nevertheless, with the molecular results of Figs. 10 and 11, it is possible to

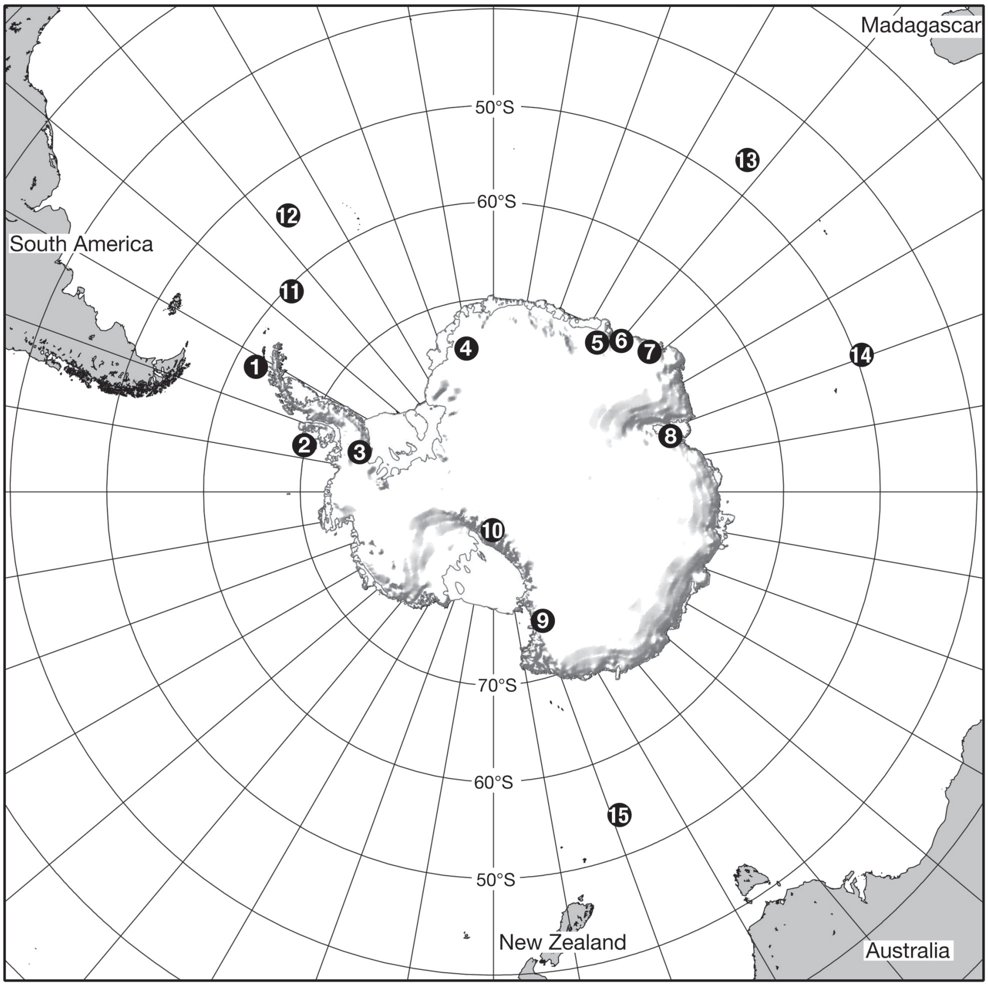

Fig. 11. Localities of *Milnesium* spp. found in and around Antarctica. **1**, King George Island; *M. antarcticum* (Tumanov, 2006). **2**, Charcot Island; MilnC-010 and MilnC-025 (Sands et al., 2008a). **3**, Ellsworth Mountains; Miln06-224 (Convey and McInnes, 2005; Sands et al., 2008b). **4**, Heimefrontfjella; *Milnesium* sp. (Sohlenius et al., 1996). **5**, Innhovde; *M. rastrum* sp. nov. **6**, Langhovde; *Milnesium* sp. (Sudzuki, 1964). **7**, Molodeznaya; possible *M. rastrum* and *Milnesium* sp. with {3-3} CC (Dastych, 1984; Utsugi and Ohyama, 1991). **8**, Larsemann Hills (Stornes Peninsula); *Milnesium* sp. Ta46-01 (Velasco-Castrillón et al., 2015). **9**, Victoria Land (Carezza Lake); *M. validum* (Pilato et al., 2017). **10**, Queen Maud Mountains; *Milnesium* sp. Ta47-1 (Velasco-Castrillón et al., 2015). **11**, Signy Island, South Orkney Islands; *Milnesium* sp. with {3-3} CC (McInnes, 1995). **12**, South Georgia; *Milnesium* sp. Miln06-108 (Sands et al., 2008b). **13**, Marion Island, Prince Edward islands; *Milnesium* sp. Miln06-123 and Miln06-124 (Sands et al., 2008b; Velasco-Castrillón et al., 2015). **14**, Kerguelen Islands; *Milnesium* sp. with "vierten Beinpaar 3/4 Krallen", i.e., $\{3\text{-}4\}^{IV}$ or $\{4\text{-}3\}^{IV}$ (Richters, 1908). **15**, Macquarie Island; *Milnesium* sp. with {3-3} CC (Miller et al., 2001). The distribution map was created using an Antarctic map (Map ID 13469, Australian Antarctic Data Centre) with a modification.

speculate that the vicinity of the Marion Hotspot might have been the center of the explosion of *Milnesium* diversity. If so, the ancestors of extant species would have dispersed from about 164 Mya, on the breakup of the Continents, West-Gondwana (Africa and South America) and East-Gondwana (Indo- Madagascar, Antarctica and Australia). Further sampling is required to prove or disprove this hypothesis.

On the signification of male *Milnesium*

The male form of *Milnesium rastrum* sp. nov. has a robust secondary claw on leg I. This characteristic claw shape was noted by Murray (1907) and Richters (1908), and then described in detail as a male-specific character (Thulin, 1928). Generally, *Milnesium* males were considered rare, with Marcus (1936) describing a single male for 25 females (Marcus, 1936), and Ramazzotti (1972) thought that there were fewer. A detailed study of male/total numbers in several populations of '*Milnesium tardigradum*' revealed: 18/75 from Sardinia, 9/26 from Buffalo Mountain Park, TN, U.S.A., 29/76 from Land Between the Lakes, TN, U.S.A., and 2/38 from New Zealand (Rebecchi and Nelson, 1998). Previously, males have been recorded for 12 species of *Milnesium*. These are: *M. beasleyi* Kaczmarek et al., 2012 from Turkey, *M. burgessi* Schilabach et al., 2018 from Kansas, U.S.A., *M. decorum* Morek et al., 2022 from Portugal, *M. dornensis* from Romania (Ciobanu et al., 2015), *M. eurystomum* Maucci, 1991 GB.005 from Scotland (Morek et al., 2020b), *M. fridae* Moreno-Talamantes et al., 2020 from Mexico, *M. inceptum* Morek et al., 2019 from Japan ('*M.* cf. *tardigradum*' in Suzuki, 2008), *M. lagniappe* Meyer et al., 2013 from Louisiana, U.S.A., *M. matheusi* from Madagascar (Kaczmarek et al., 2019), *M. swansoni* Young et al., 2016 from Kansas, U.S.A., *M. tetralamellatum* Pilato and Binda, 1991 from Tanzania, and *Milnesium* sp. (MilnC_010) from Charcot Is, Antarctica (Sands et al., 2008a, additional file 1); the last of which may be *M. antarcticum. Milnesium rastrum* sp. nov. is the latest addition to this list.

It is unknown whether any species in the above list have facultative parthenogenesis. Although *M. inceptum* always reproduces without males, male individuals emerged at a very low frequency even in such a thelytokous population (Suzuki, 2008); the reason for this has not yet been elucidated. The first author has observed that individuals of *M. inceptum*, since 2000, have laid at least one egg during the moulting period, even if the nutritional condition was not good. On the other hand, the solitary female of *M. rastrum* sp. nov. did two cycles of moult without oviposition, suggesting an obligate mode of bisexual reproduction. In dioecious tardigrades, females isolated from males never lay eggs (Lemloh et al., 2011; Bingemer et al., 2016). The same condition was apparently observed in *M. eurystomum* GB.005, in which males were found, and described as follows: “Since mature females extracted from the GB.005 sample did not lay additional eggs, the culture was terminated after a short period of time” (Morek et al., 2020b).

While parthenogenesis is considered more common in harsh environments (Nelson et al., 2015), it is interesting to note that an isolated ice-free area like Innhovde contains the presumed non-parthenogenetic species *M. rastrum* sp. nov. This may account for the rarity of *Milnesium* over the East Antarctica area, and suggests that *M. rastrum* sp. nov. had retained the plesiomorphic trait of bisexual reproduction since the isolation of Antarctica.

Tardigrade dispersal mechanisms are relatively poorly studied and the exact means are unknown. In the genus *Ramazzottius* Binda and Pilato, 1987, which has both reproductive modes, the diploid bisexual populations were found mostly in large moss or lichen encrustations on rocky outcrops, while the triploid or tetraploid parthenogenetic populations were from newly formed patches of moss or lichen on tree trunks (Bertolani et al., 1990). This observation suggests a theory that parthenogenesis with multiploidy originated somewhere, and then settled and propagated in harsh and isolated habitats. However, this theory cannot be applied to the genus *Milnesium* because parthenogenetic populations of *M. tardigradum*, *M. pacificum* Sugiura et al., 2020, and *M. inceptum* all showed diploid chromosomes (Sugiura et al., 2020). So, parthenogenetic *Milnesium* flourish in harsh environments regardless of the ploidy.

Antarctica was a warm and well-vegetated land mass before becoming isolated and frozen about 30 Mya. Tardigrades, able to survive the harsher environment, would have survived, staying in the remaining vegetation but becoming more isolated by glacier formations. This, and the more plesiomorphic bisexual reproduction as the ancestral mode may account for the isolated pockets of Antarctic dioecious tardigrades, e.g., *M. rastrum* sp. nov., *Mopsechiniscus franciscae* Guidetti et al., 2014, (Victoria Land), and *Echiniscus corrugicaudatus* McInnes, 2010 (inland nunataks in Ellsworth Land). While these Antarctic environments are extremely isolated, they have also provided very stable and rich habitats as refugia for tiny bisexual creatures for several tens of millions of years. However, this also makes these ecosystems very fragile against settlement of more fertile parthenogenetic animals as well as contemporary invaders (see Hughes et al., 2020) or possible future destruction of the natural barrier around the Antarctic Continent (Convey and Peck, 2019).

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COMPETING INTERESTS

The authors have no competing interest to declare.

AUTHOR CONTRIBUTIONS

ACS, MT, and RN did the fieldwork in the ice-free area at Innhovde. SI supervised the activities in Antarctica and identified the moss species. ACS and MT examined the sample to collect tardigrade specimens. ACS designed the study including tardigrade culture and prepared the draft of the text and most figures. HK and KS performed the DNA analyses. KS performed phylogenetic analyses and prepared text and figures of phylogenetic trees. All authors read and approved the manuscript.

SUPPLEMENTARY MATERIALS

Supplementary materials for this article are available online. (URL: https://doi.org/10.2108/zs220085)

Supplementary Figure S1. Maximum likelihood phylogenetic trees based on concatenated (18S + 28S + ITS-2 + COI) sequence.

Supplementary Table S1. DNA sequences used for analyses.

REFERENCES

Bertolani R, Rebecchi L, Beccaccioli G (1990) Dispersal of *Ramazzottius* and other tardigrades in relation to type of reproduction. Invertebr Reprod Dev 18: 153–157

Binda MG, Pilato G (1987) *Ramazzottius*, nuovo genere di Eutardigrado (Hypsibiidae). Animalia (Catania) 13: 159–166

Bingemer J, Hohberg K, Schill RO (2016) First detailed observations on tardigrade mating behaviour and some aspects of the life history of *Isohypsibius dastychi* Pilato, Bertolani & Binda 1982 (Tardigrada, Isohypsibiidae). Zool J Linn Soc 178: 856–862

Blaxter ML, De Ley P, Garey JR, Liu LX, Scheldeman P, Vierstraete A, et al. (1998) A molecular evolutionary framework for the phylum Nematoda. Nature 392: 71–75

Bryce D (1892) On some moss-dwelling Catypnadæ; with descriptions of five new species. Hardwicke’s Sci-Gossip 28: 325–336

Camarda D, Pilato G, Lisi O (2022) Considerations on the claws of the Apochela and a novel detail of the bucco-pharyngeal apparatus of the genus *Milnesium* (Tardigarda: Apochela: Milnesiidae). Eur Zool J 89: 256–277

Ciobanu D, Zawierucha K, Moglan I, Kaczmarek Ł (2014) *Milnesium berladnicorum* sp. n. (Eutardigrada, Apochela, Milnesiidae), a new species of water bear from Romania. ZooKeys 429: 1–11

Ciobanu DA, Roszkowska M, Kaczmarek Ł (2015) Two new tardigrade species from Romania (Eutardigrada: Milnesiidae, Macrobiotidae), with some remarks on secondary sex characters in *Milnesium dornensis* sp. nov. Zootaxa 3941: 542–564

Convery P, McInnes SJ (2005) Exceptional tardigrade-dominated ecosystems in Ellsworth Land, Antarctica. Ecology 86: 519–527

Convey P, Peck LS (2019) Antarctic environmental change and biological responses. Sci Adv 11: eaaz0888

Dastych H (1984) The Tardigrada from Antarctic with descriptions of several new species. Acta Zool Cracov 27: 377–436, pls XII–XVII

Doyère LMF (1840) Memoire sur les Tardigrades. I. Ann Sci Nat Sér 2 14: 269–362

Ehrenberg CG (1853) Diagnoses novarum formarum. Ber Bekanntm Verh K Preuss Akad Wiss Berlin 8: 526–533

Ehrenberg CG (1854) Mikrogeologie. Atlas. Tafel 35B. Voss, Leipzig

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3: 294–299

Gąsiorek P, Stec D, Morek W, Michalczyk Ł (2017) An integrative redescription of *Echiniscus testudo* (Doyère, 1840), the nominal taxon for the class Heterotardigrada (Ecdysozoa: Panarthropoda: Tardigrada). Zool Anz 270: 107–122

Guidetti R, Rebecchi L, Cesari M, McInnes SJ (2014) *Mopsechiniscus franciscae*, a new species of a rare genus of Tardigrada from continental Antarctica. Polar Biol 37: 1221–1233

Guidetti R, McInnes SJ, Cesari M, Rebecchi L, Rota-Stabelli O (2017) Evolutionary scenarios for the origin of an Antarctic tardigrade species based on molecular clock analyses and biogeographic data. Contrib Zool 86: 97–110

Hughes KA, Pescott OL, Peyton J, Adriaens T, Cottier-Cook EJ, Key G, et al. (2020) Invasive non-native species likely to threaten biodiversity and ecosystems in the Antarctic Peninsula region. Glob Change Biol 26: 2702– 2716

Kaczmarek Ł, Jakubowska N, Michalczyk Ł (2012) Current knowledge on Turkish tardigrades with a description of *Milnesium beasleyi* sp. nov. (Eutardigrada: Apochela: Milnesiidae, the *granulatum* group). Zootaxa 3589: 49–64

Kaczmarek Ł, Grobys D, Kulpa A, Battylak T, Kmita H, Kepel M, et al. (2019) Two new species of the genus *Milnesium* Doyère, 1840 (Tardigrada, Apochela, Milnesiidae) from Madagascar. ZooKeys 4: 1–22

Kagoshima H, Imura S, Suzuki AC (2013) Molecular and morphological analysis of an Antarctic tardigrade, *Acutuncus antarcticus*. J Limnol 72(s1): 15–23

Katoh K, Toh H (2008) Recent developments in the MAFFT multiple sequence alignment program. Brief Bioinform 9: 286–298

Katoh K, Misawa K, Kuma K, Miyata T (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res 30: 3059–3066

Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol 33: 1870–1874

Lemloh M-L, Brümmer F, Schill RO (2011) Life-history traits of the bisexual tardigrades *Paramacrobiotus tonollii* and *Macrobiotus sapiens*. J Zool Syst Evol Res 49(S1): 58–61

Maucci W (1991) Tre nuove specie di Eutardigradi della Groenlandia Meridionale. Boll Mus Civ Stor Nat Verona 15: 279–289

Marcus E (1936) Tardigrada. Das Tierreich, 66. Walter de Gruyter, Berlin und Leipzig

McDougall I, Verwoerd W, Chevallier L (2001) K–Ar geochronology of Marion Island, Southern Ocean. Geol Mag 138: 1–17

McInnes SJ (1995) Tardigrades from Signy Island, South Orkney Islands, with particular reference to freshwater species. J Nat Hist 29: 1419–1445

McInnes SJ (2010) *Echiniscus corrugicaudatus* (Heterotardigrada; Echiniscidae) a new species from Ellsworth Land, Antarctica. Polar Biol 33: 59–70

McInnes SJ, Pugh PJA (1998) Biogeography of limno-terrestrial Tardigrada, with particular reference to the Antarctic fauna. J Biogeogr 25: 31–36

Meyer HA, Hinton JG, Dupré MC (2013) *Milnesium lagniappe*, a new species of water bear (Tardigrada, Eutardigrada, Apochela, Milnesiidae) from the Southern United States. West N Am Nat 73: 295–301

Michalczyk Ł, Wełnicz W, Frohme M, Kaczmarek Ł (2012) Redescriptions of three *Milnesium* Doyère, 1840 taxa (Tardigrada: Eutardigrada: Milnesiidae), including the nominal species for the genus. Zootaxa 3154: 1–20

Miller WR, Horning DS, Heatwole HF (2001) Tardigrades of the Australian Antarctic: Macquarie Island, sub-Antarctica. Zool Anz 240: 475–491

Minh BQ, Schmidt HA, Chernomor O, Schremph D, Woodhams MD, von Haeseler A, et al. (2020) IQ-TREE 2: new models and efficient methods for phylogenetic inference in the genomic era. Mol Biol Evol 37: 1530–1534

Mironov SV, Dabert J, Dabert M (2012) A new feather mite species of the genus *Proctophyllodes* Robin, 1877 (Astigmata: Proctophyllodidae) from the Long-tailed Tit *Aegithalos caudatus* (Passeriformes: Aegithalidae)—morphological description with DNA barcode data. Zootaxa 3253: 54–61

Morek W, Suzuki AC, Schill RO, Georgiev D, Yankova M, Marley NJ, et al. (2019) Redescription of *Milnesium alpigenum* Ehrenberg, 1853 (Tardigrada: Apochela) and a description of *Milnesium inceptum* sp. nov., a tardigrade laboratory model. Zootaxa 4586: 35–64

Morek W, Ciosek JA, Michalczyk Ł (2020a) Description of *Milnesium pentapapillatum* sp. nov., with an amendment of the diagnosis of the order Apochela and abolition of the class Apotardigrada (Tardigrada). Zool Anz 288: 107–117

Morek W, Blagden B, Kristensen RM, Michalczyk Ł (2020b) The analysis of inter- and intrapopulation variability of *Milnesium eurystomum* Maucci, 1991 reveals high genetic divergence and a novel type of ontogenetic variation in the order Apochela. Syst Biodivers 18: 614–632

Morek W, Surmacz B, López A, Michalczyk Ł (2021) ‘Everything is not everywhere’: time-calibrated phylogeography of the genus *Milnesium* (Tardigrada). Mol Ecol 30: 3590–3609

Morek W, Wałach K, Michalczyk Ł (2022) Rough backs: taxonomic value of epicuticular sculpturing in the genus *Milnesium* Doyère, 1840 (Tardigrada: Apochela). Sci Rep 12: 9857

Moreno-Talamantes A, León-Espinosa GA, García-Aranda MA, Flores-Maldonado JJ, Kaczmarek Ł (2020) The genus *Milnesium* Doyère, 1840 in Mexico with description of a new species. Ann Zool (Wars) 70: 457–486

Mueller CO, Jokat W (2019) The initial Gondwana break-up: A synthesis based on new potential field data of the Africa-Antarctica Corridor. Tectonophysics 750: 301–328

Murray J (1907) Scottish Tardigrada. Trans R Soc Edinb 45: 641–668, pls I–IV

Nederström P (1919) Die bis jetzt aus Finnland bekannten Tardigraden. Acta Soc Fauna Flora Fenn 46: 1–25

Nelson DR, Guidetti R, Rebecchi L (2015) Phylum Tardigrada. In “Thorp and Covich’s Freshwater Invertebrates. 4th ed.” Ed by JH Thorp, DC Rogers, Academic Press, Boston, pp 347–380

Pilato G (1981) Analisi di nuovi caratteri nello studio degli eutardigradi. Animalia (Catania) 8: 51–57

Pilato G, Binda MG (1991) *Milnesium tetralamellatum*, new species of Milnesiidae from Africa (Eutardigrada). Trop Zool 4: 103–106

Pilato G, Savella G, D’Urso V, Lisi O (2017) Two new species of Eutardigrada from Victoria Land, Antarctica. Zootaxa 4317: 541–558

Ramazzotti G (1962) Tardigradi del Cile–con descrizione di quattro nuove specie e di una nuova varietà. Atti Soc Ital Sci Nat Mus Civ Stor Nat Milano 101: 275–287

Ramazzotti G (1972) Il Phylum Tardigrada. Mem Ist Ital Idrobiol 28: 1–732

Rebecchi L, Nelson DR (1998) Evaluation of a secondary sex character in eutardigrades. Invertebr Biol 117: 194–198

Richters F (1902) Neue Moosbesohner. Ber Senckenb Naturforsch

Ges Frankf 1902(2): 23–26

Richters F (1904) Arctische Tardigraden. Fauna Arct 3: 493–508

Richters F (1908) Die Fauna der Moosrasen des Gaussbergs und Einiger Südlicher Inseln. In "Deutsche Südpolar-Expedition, 1901–1903, Bd. 9 (Zoologie I), Heft 4 (1907)" Ed by E von Drygalski, G. Reimer, Berlin, pp 259–302, taf 16–20

Richters F (1926) Tardigrada. Handb Zool (Berl) 3: 58–61

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574

Ronquist F, Teslenko M, Mark P, Ayres DL, Darling A, Höhna S, et al. (2012) MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol 61: 539–542

Sands CJ, Convery P, Linse K, McInnes SJ (2008a) Assessing meiofaunal variation among individuals utilising morphological and molecular approaches: an example using the Tardigrada. BMC Ecol 8: 7

Sands CJ, McInnes SJ, Marley NJ, Goodall-Copestake WP, Convey P, Linse K (2008b) Phylum Tardigrada: an "individual" approach. Cladistics 24: 861–871

Schilabach S, Donaldson E, Hobelman K, Miller WR, Lowman MD (2018) Tardigrades of the canopy: *Milnesium burgessi* nov. sp. (Eutardigrada: Apochela: Milnesiidae) a new species from Kansas, U.S.A. Trans Kans Acad Sci 121: 39–48

Schuster RO, Nelson DR, Grigarick AA, Christenberry D (1980) Systematic criteria of the Eutardigrada. Trans Am Microsc Soc 99: 284–303

Sohlenius B, Boström S, Hirschfelder A (1996) Distribution patterns of microfauna (nematodes, rotifers and tardigrades) on nunataks in Dronning Maud Land, East Antarctica. Polar Biol 16: 191–200

Storey M, Mahoney JJ, Saunders AD, Duncan RA, Kelley SP, Coffin MF (1995) Timing of hot spot-related volcanism and the breakup of Madagascar and India. Science 267: 852–855

Sudzuki M (1964) On the microfauna of the Antarctic region I. Moss-water community at Langhovde. JARE 1956–1962 Sci Rep Ser E 19: 1–41, pls I–XI

Sugiura K, Minato H, Matsumoto M, Suzuki AC (2020) *Milnesium* (Tardigrada: Apochela) in Japan: The first confirmed record of *Milnesium tardigradum* s.s. and description of *Milnesium pacificum* sp. nov. Zool Sci 37: 476–495

Suzuki AC (2003) Life history of *Milnesium tardigradum* Doyère (Tardigrada) under a rearing environment. Zool Sci 20: 49–57

Suzuki AC (2008) Appearance of males in a thelytokous strain of *Milnesium* cf. *tardigradum* (Tardigrada). Zool Sci 25: 849–853

Suzuki AC (2022) Beautiful claws of a tiny water bear: a review and proposal for claw configuration. Zool Sci 39: 167–175

Thulin G (1928) Über die Phylogenie und das System der Tardigraden. Hereditas 11: 207–266

Torsvik TH, Tucker RD, Ashwal LD, Eide EA, Rakotosolofo NA, de Wit MJ (1998) Late Cretaceous magnetism in Madagascar: palaeomagnetic evidence for a stationary Marion hotspot. Earth Planet Sci Lett 164: 221–232

Tumanov DV (2006) Five new species of the genus *Milnesium* (Tardigrada, Eutardigrada, Milnesiidae). Zootaxa 1122: 1–23

Utsugi K, Ohyama Y (1991) Antarctic Tardigrada II. Molodezhnaya and Mt. Riiser-Larsen areas. Proc NIPR Symp Polar Biol 4: 161–170

Velasco-Castrillón A, McInnes SJ, Schultz MB, Arróniz-Crespo M, D'Haese CA, Gibson JAE, et al. (2015) Mitocondrial DNA analyses reveal widespread tardigrade diversity in Antarctica. Invertebr Syst 29: 578–590

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In "PCR protocols: A Guide to Methods and Application" Ed by MA Innes, DH Gelfand, JJ Sninsky, TJ White, Academic Press, San Diego, pp 315–322

Young A, Chappell B, Miller WR, Lowman M (2016) Tardigrades of the tree canopy: *Milnesium swansoni* sp. nov. (Eutardigrada: Apochela: Milnesiidae) a new species from Kansas, U.S.A. Zootaxa 4072: 559–568

Zhang J, Kapli P, Pavlidis P, Stamatakis A (2013) A general species delimitation method with applications to phylogenetic placements. Bioinformatics 29: 2869–2876

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